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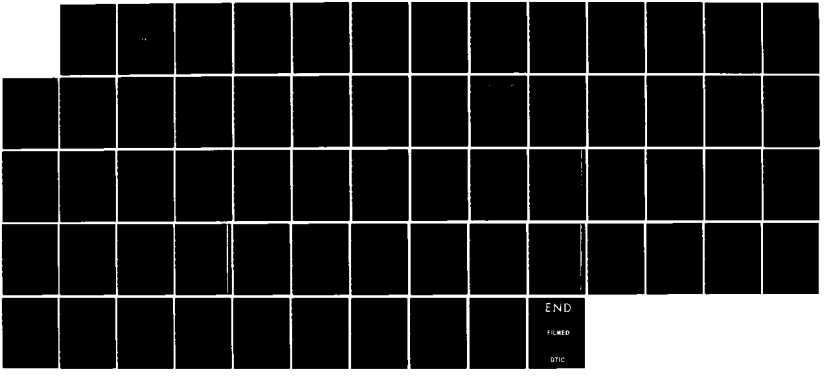

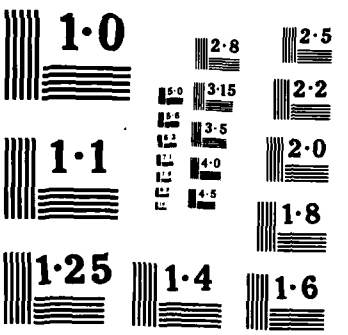
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International Workshop on the Molecular Biology of Flaviviruses

November 29 - December 1, 1984

U.S. Army Medical Research Institute of Infectious Diseases

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

An International Workshop on the Molecular Biology of Flaviviruses was held at the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland, on 29 November to 1 December 1984. The objectives of this workshop were to examine the current status of the molecular virology of these agents and develop concepts for future flavivirus vaccine development. This document constitutes the proceedings of that symposium. Cont'd on next page

Preface

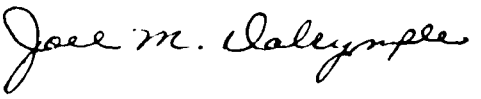
A-1

The U.S. Army Medical Research and Development Command (USAMRDC) has had a long-term research interest in flaviviruses because of their demonstrated potential for causing large epidemics of serious human disease in many parts of the world. The goal of this research has been to protect U.S. personnel stationed abroad, as well as to provide relief to major foreign populations suffering from endemic disease. Much of the research has been conducted in Washington, D.C., at the Walter Reed Army Institute of Research (WRAIR) and at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), in direct support of and in close collaboration with overseas laboratories in endemic disease areas such as the Armed Forces Research Institute for Medical Sciences (AFRIMS) in Bangkok, Thailand. In addition to this in-house effort, USAMRDC has sponsored a contract-funding program for extramural research that historically has emphasized research on members of the Flaviviridae. The Centers for Disease Control (CDC), the World Health Organization (WHO), and the U.S.-Japan Panel for Cooperative Medical Research have also contributed significantly to research on flaviviruses. However, it is generally accepted that both existing research efforts and current funding programs are quite modest in light of the magnitude of the global disease problems.

The stimuli for conducting an International Workshop on the Molecular Biology of Flaviviruses were numerous. First, molecular gene cloning technology has opened new horizons for the study of flaviviruses, and it seemed prudent and practical to compare notes at the working level and to prepare others for the ultimate testing of gene-cloned products of immunogenic potential. Second, the time between observation and interpretation of experimental results and publication in an international journal is too long for anyone wishing to stay abreast of the most recent developments in the field. Further, flaviviruses represent a unique group of agents with a replication strategy and set of problems for the researcher that are quite different from Togaviridae. This fact was recently recognized by the International Committee on the Taxonomy of Viruses as it assigned family status to Flaviviridae. Finally, the number of investigators in this area is relatively small with little incentive to attract the fresh approach of young investigators into this field of slow-growing and low-titered viruses, difficult research problems, and limited prospects for research funding. Although funding constraints did not allow travel support for a large number of students or junior colleagues to this workshop, it was refreshing to see numerous newcomers to the field as both spectator and participant.

Flavivirologists have long been divided by discipline into those whose primary interest is in the laboratory aspects of virus research, field ecologists, epidemiologists, and the entomologists studying the natural vectors of these viruses. Most successful flavivirus research has been the result of a team effort combining the efforts of each of these subdisciplines. This workshop was designed to emphasize the more molecular laboratory aspects, since all facets of the disease problem could not be consolidated into a small meeting of this type. It is hoped that these other aspects of Flaviviridae research and disease control will become the subjects of future workshops.

As this booklet of proceeding abstracts is being readied for distribution, some of the benefits of this workshop are already in evidence. There is an obvious increase in the research interest in these viruses and a significant improvement in communication between investigators. Both in-house and extramural USAMRDC investigators have adjusted the focus of their programs and have made numerous friends of others with similar interests and problems in the academic virology community. The workshop sponsors at USAMRIID and USAMRDC would like to take this opportunity to thank all participants who gave so freely of their time and recent data to help make this workshop a stimulating, scientific experience.



Joel M. Dalrymple, Ph.D.
Chief, Department of Viral Biology
Virology Division, USAMRIID

Contents

INTERNATIONAL WORKSHOP ON THE MOLECULAR BIOLOGY OF FLAVIVIRUSES

U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID)
Fort Detrick, Frederick, Maryland 21701 U.S.A.

November 29 - December 1, 1984

Agenda

Thursday, November 29

7:00 pm	Introductory Evening Session REGISTRATION/Cash Bar	Sheraton Inn-Frederick
8:00	Welcome	COL David L. Huxsoll Commander, USAMRIID
	Flavivirus Disease Problems: A Perspective	Tom Monath
	Agenda and Workshop Plans	Joel Dalrymple
9:30	Social Hour	
10:30	ADJOURN	

Friday, November 30

8:00 am	REGISTRATION	Conference Room 120 USAMRIID
	I. Flavivirus Replication Strategy Chairpersons: James Strauss, E.G. Westaway	
8:30	Molecular Cloning and Nucleotide Sequence Analysis of Genomic RNA of St. Louis Encephalitis Virus	Vance Vorndam
	Genetic Variation Among and Within Dengue Virus Serotypes	Jan Blok
	Molecular Cloning and Structural Analysis of Dengue Virus Type 2 Genome	Radha Padmanabhan
	BREAK	

Friday, November 30

	Molecular Cloning and Genomic Organization of Japanese Encephalitis Virus	Skip Fournier
	Structure of the Yellow Fever Virus Genome	Charles Rice
	ROUND-TABLE DISCUSSION (1 hour)	Drs. Ellen Strauss, Dennis Trent, Ching-Juh Lai, Vic Stollar, Margo Brinton, and Carol Blair
12:00 pm	LUNCH	

II. Flavivirus Immunology - Antigen/Antibody Characterization
Chairpersons: Walt Brandt, James Porterfield

1:30 pm	Molecular Immunochemistry of St. Louis Encephalitis Virus: Antigenic Structure, Immunity, and Artificial Immunogens	John Roehrig
	Antigenic Structure of Japanese Encephalitis Virus	Kotaro Yasui
	Epitope Mapping of the Envelope Glycoprotein of Japanese Encephalitis Virus	Tom Mason
	BREAK	
	Functional and Immunochemical Characterization of Antigenic Determinants on the Tick-Borne Encephalitis Virus Glycoprotein	Franz Heinz
	Immune Mechanisms in Experimental Yellow Fever Virus Infection	Jack Schlesinger
	Dengue Epitope Mapping: Comparison of the Four Dengue Serotypes	Don Burke
4:00 pm	ROUND-TABLE DISCUSSION (1 hour)	Drs. Jim Mathews, Gerry Cole, Alan Schmaljohn, Srisakul Kliks, and Mary Kay Gentry
5:00 pm	ADJOURN	
7:00 pm	BANQUET	Sheraton Inn

aturday, December 1

III. Future Vaccine Strategy
Chairpersons: Walter Schlesinger, Philip Russell

:00 am	Currently Available Vaccines for Japanese Encephalitis and Strategies for Their Improvement	Robert Shope
	Yellow Fever Vaccine: Rationales and Approaches to Modernization	Tom Monath
	BREAK	
	Theoretical and Practical Considerations for Dengue Vaccination	Ken Eckels
	Designing New Vaccines Against RNA Viruses	John Stephenson
:00 pm	LUNCH	
.:00 pm	ROUND-TABLE DISCUSSION (1 hour)	Drs. Fred Murphy, Robert Chanock, Gerry Eddy, Sutee Yoksan, Vina Churdboonchart, Fakhry Assaad, Natth Bhamarapravati, and Scott Halstead
	CLOSING REMARKS	
:00 pm	ADJOURN	

Introduction
Medical Importance of the Flaviviruses

TITLE: Medical Importance of the Flaviviruses

THOR: Thomas P. Monath, M.D.

DRESS: *Division of Vector-Borne Viral Diseases, Centers for Disease Control,
P.O. Box 2087, Fort Collins, CO 80522-2087
LEPHONE: 303-221-6400

The Flaviviridae comprise a family of 60 viruses, of which 27 cause human disease and 10, including 7 mosquito-borne and 3 tick-borne viruses, are associated with significant morbidity and mortality. Some of the mosquito-borne viruses, such as dengue, yellow fever, West Nile and Japanese encephalitis, have wide geographic distributions in subtropical and tropical areas, whereas other agents associated with mosquito vectors in the temperate zone or with tick vectors have more restricted distribution. Only 4 flaviviruses (Japanese encephalitis, Wesselsbron, louping ill, and Israel turkey meningo-encephalitis) are clearly implicated as veterinary pathogens. The predominant disease patterns produced in man and domestic livestock may be classified as neuromorphic (meningitis, encephalitis), visceromorphic (hepatitis, hemorrhagic fever), or pantamorphic (nondescript febrile illness, febrile exanthem, etc.), but a significant degree of overlap exists between the patterns produced by individual viruses. In terms of global morbidity, dengue leads, with between several hundred thousand and several million cases annually. Yellow fever and Japanese encephalitis follow, being responsible for thousands to tens of thousands of cases per year. Tick-borne encephalitis and Kyasanur forest disease cause hundreds to several thousands of clinical infections annually. All of these diseases are under-reported. Case-fatality rates vary widely, but may be as high as 20 percent (yellow fever) to 30 percent (Russian spring summer encephalitis). The encephalitic infections often result in neuropsychiatric sequellae, further increasing the burden of these diseases on society.

The pathogenesis of flavivirus infections is incompletely understood. Both virus-specified and host-determined factors play important roles in disease expression. Plasticity of the viral genome and variability and heterogeneity of natural virus populations will be discussed again in this symposium, but there is abundant evidence for circulation of virus strains with varying virulence. Flaviviruses demonstrate a wide spectrum of tissue tropisms, but the molecular basis of virus-cell receptor interactions has not been elucidated. Genetic control of flavivirus susceptability/resistance has long been known in the mouse model; resistance appears to be associated with increased production of defective interfering particles, but again the molecular mechanisms involved remain uncertain. Immunosuppression-reconstitution experiments have shown both cell-mediated and humoral responses to be involved in recovery from primary infection, but antibody probably plays a predominant role. The disease state comprises not only direct viral cytolysis but also immunopathologic damage mediated by antibody, complement, and lymphoid cells. A clearer definition of the immune responses to flavivirus infections and the viral antigens specifying these responses is critical to our understanding of pathogenesis as well as disease prevention through vaccination or immunomodulation.

Control of flavivirus infections is still oriented largely toward reduction or elimination of arthropod vectors--an approach which has met with limited successes in the past but promises increasing difficulties in the future. Antiviral chemotherapy has not yet provided useful leads toward the control of flaviviral infections. Vaccines appear to be within reach as effective means for control, and represent the major thrust of research on the molecular biology of these agents.

*Until October 1985: Gastroenterology Unit, Massachusetts General Hospital,
Boston, MA 02114 (617) 726-3766

TITLE: Molecular immunochemistry of St. Louis encephalitis virus: antigenic structure, immunity and artificial immunogens.

AUTHORS: J.T. Roehrig[1], J.H. Mathews[1], A.R. Hunt[1], L.A. Staudinger[1], and C.D. Blair[2].

ADDRESS: [1]DVBVD, CDC, P. O. Box 2087, Ft. Collins, CO. 80522 USA.
[2]Dept. Microbiology, Colorado State Univ., Ft. Collins, CO., 80523 USA.

TELEPHONE: [1] 303-221-6400 and [2] 303-491-6810.

St. Louis encephalitis (SLE) virus is the etiologic agent for a severe encephalitis in the very young and the very old. We have isolated monoclonal antibodies (MAbs) specific for the E-glycoprotein of SLE virus (MSI-7), and used them to analyze the antigenic structure of this protein. Serologic reactivities of these MAbs were initially determined by cross-reactivity indirect immunofluorescence assay (IFA) using 2 strains of SLE virus and eight related flaviviruses. Four groups demonstrating type-, subcomplex-, subgroup-, and group-specific patterns were identified. Analysis of hemagglutination-inhibition and neutralization (N) activity subdivided the cross-reactivity groups into eight epitopes. The type-specific MAbs could detect antigenic drift among SLE virus strains isolated from various geographic regions. Analysis of antigenic reactivity of these epitopes in immunoblot after boiling in 2% SDS and 3% β-mercaptoethanol indicated that all but the E-1^d epitope appeared conformationally stable. Expression of these epitopes on the surface of virus-infected Vero cells could be separated into two groups. The first group of these epitopes could be detected by IFA on unfixed target cells at eight hours post-infection. The second group was detected after 16 hours post-infection. It is yet unknown whether this differential expression is due to protein processing. Competitive binding assay (CBA) with representative MAbs of similar binding avidities, indicated that the glycoprotein was a continuum of six overlapping domains.

The E-1^c epitope encoded for the type-specific biologic functions of hemagglutination and N. Injection of 50 ng of anti-E-1^c MAb protected the majority of mice from a lethal peripheral challenge with 100 i.p. LD_{50} of SLE virus. Similar levels of protection with MAbs specific for other epitopes could be attained if 1000-fold more MAb was used. Attempts to block N or protection mediated by anti-E-1^c MAb using other MAbs that interfered with anti-E-1^c MAb binding in CBA were unsuccessful. Enhancement of protection could be demonstrated when mixtures of non-neutralizing, cross-reactive MAbs were used in passive protection. The E-1^c MAb was also effective in abrogating SLE virus induced disease if administered prior to neural invasion of virus.

The well documented phenomenon of cross-protection from various flaviviral diseases was investigated using these MAbs and three other MAbs isolated from fusions with Japanese encephalitis (JE) virus immunized mice. Mice were passively immunized with various cross-reactive MAbs, and were then challenged with a lethal peripheral inoculation of either SLE, JE, or Murray Valley encephalitis (MVE) viruses. These results indicate that there were several cross-reactive [illegible] E-glycoprotein which had differing protective capacities. No one MAb protected from all virus challenges. In general, the high avidity MAbs protected [illegible] low avidity MAbs. Protection was most efficient when MVE virus was [illegible] mice. Protection was similar for SLE and JE virus challenges [illegible] survival time was significantly increased with most of these antibodies [illegible] challenge virus.

Anti-E-1^c and anti-E-4^b MAbs were used to elicit rabbit [illegible] (id) antibody. Rabbits were immunized with purified MAb and boosted with a mixture of Fab and $F(ab')_2$ fragments. Rabbit anti-mouse IgG cross-reactive antibody was removed by absorption to an affinity column prepared from MAb of like subclass (IgG 2a) but different specificity. We have been able to demonstrate that id-anti-id reaction can be blocked by addition of virus. The virus-id reaction can also be blocked by preincubation of anti-id with id. Anti-id was specific for E-1^c id because it failed to recognize other SLE virus MAbs. Immunization of mice with anti-id in an attempt to anti-viral antibody is in progress.

II. Flavivirus Immunology — Antigen/Antibody Characterization

changes might be expected to be selected against, but sequencing experience has shown that even noncoding changes do not accummulate rapidly upon passage, suggesting that such changes are also selected against. Such selection could occur if RNA secondary structure was important for the most efficient RNA replication and packaging.

Dr. Trent followed this up by noting that given mutability of RNA genomes it was remarkable that field isolates of both alpha and flaviviruses appeared to change so slowly (in particular, dengue isolates in the last 20 years) and suggested that other forces which we do not understand were slowing down the evolution of these viruses in a natural setting.

The desirability of identifying epitopes on the E protein for use in immunization was discussed by Dr. J. Schlesinger. However, attempts to obtain suitable peptide fragments were hampered by problems in obtaining adequate amounts of purified E.

Further support for the concept that flavivirus proteins are produced by post-translational processing of larger precursors was presented in the Saturday session by Dr. C. Blair. She has found in lysates of JE-infected cells labeled by short pulses that large proteins of >200 K (which is larger than the largest nonstructural protein recognized to date), 95 K and 81 K are specifically immunoprecipitated by anti-JE antiserum and that by limited digestion with V8 protease the larger polypeptide appears to share peptides with the smaller proteins of 55 K, 41 K, 29 K and 20 K. These large proteins are most prominent after very short pulses (as little as 30 sec) and appear to chase in pulse-chase experiments. Dr. Stollar commented that Dr. G. Cleaves finds similar precursors in dengue infected cells treated with protease inhibitors such as zinc and TPCK, and Dr. Vorndam also cited the appearance of larger proteins in SLE-infected cells. Dr. E. Westaway mentioned that small amounts of larger products were occasionally seen in experiments with Kunjin virus, but these were not reproducible; his colleague, Dr. Wright, had observed some larger products produced in very small amounts in the presence of some inhibitors.

Returning to the discussion in the Friday session, Dr. Stoller asked if comparison of the sequences of the glycoproteins of viruses (in particular, of alphaviruses which bud from the plasmalemma and of flavi- and coronaviruses which bud internally) revealed any features which could be correlated with the sites of budding. Dr. J. Strauss said there was no data bearing on this but both Dr. Westaway and Dr. Vorndam mentioned that flaviviruses, perhaps because of their mode of maturation, incorporated unglycosylated as well as glycosylated forms of the glycoprotein E into their virions. Dr. Vorndam further stated that SLE envelope proteins do not contain covalently attached lipids.

In response to a query from J. Strauss, Dr. Monath commented on the different lineages of yellow fever vaccine strains.

Returning to the problem of sharing sequence information to accelerate progress in cloning and sequencing of flaviviruses Dr. Dalrymple asked if there was possibility of "universal flavivirus primers" from current sequences. Dr. Rice felt that there might be consensus sequences in the 3' untranslated but felt that primers for diagnosis or sequencing in the glycoprotein difficult due to divergence of the various viruses as exemplified variation. Dr. Blok commented that she had used a synthetic consensus sequence of the 3' terminus of West Nile virus to prime New Guinea C dengue 2 RNA but that it failed to work with perhaps for technical reasons.

Following up on this point, Dr. Strauss pointed out that coding regions which could be useful would be degenerate sequences since RNA viruses in general have been found to a particular conserved amino acid during evolution. This comparative study of alphaviruses where an amino acid sequence served and yet the nucleotide sequences show no more than third position of the codons. Therefore even in regions of flaviviruses one would not expect conservation at the nucleotide make specific primers usable. Dr Wimmer agreed and pointed out strains 1, 2 and 3 have a small number of amino acid restriction map in common and minimal homology at the RNA level.

Dr. Strauss commented that because of the high mutation rate in the order of 10^{-4} per nucleotide per generation, any virus RNA a population of molecules which possesses an average sequence molecules differ in one or more positions from this average. perhaps surprising that RNA virus strains show as much stability as they do.

TITLE: Summary of Round Table Discussion Following Session I - Flavivirus Replication Strategy

AUTHORS: J. H. Strauss and E. Westaway

ADDRESS: Division of Biology, California Institute of Technology, Pasadena, CA 91125

TELEPHONE: (818) 356-4903

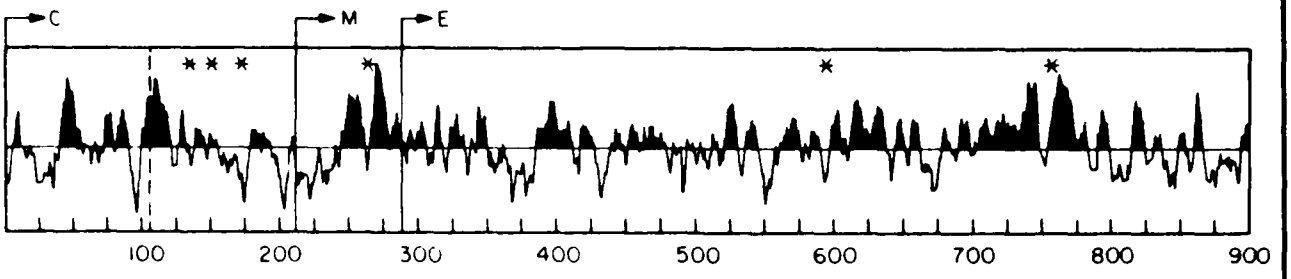
Three of the presentations had dwelled upon the possible structures and sequences at the 3' terminus of flavivirus RNA and the effect of this structure on priming, especially for cDNA synthesis preparatory to cloning of the flavivirus genomes. It has been reported in the literature 1) that flavivirus genomic RNAs lack a poly(A) tail and 2) that it is extremely difficult to label or modify the 3' end of some flavivirus RNAs.

The first point of discussion was the observation by Dr. Blok that cDNA to DEN 1 and DEN 2 RNAs could be synthesized effectively using an oligo(dT) primer. Dr. Trent pointed out that when he examined various strains of dengue virus by T1 oligonucleotide mapping no oligonucleotide with more than 10 A's was found. Dr. Rice observed that pure oligo(A) was probably not required and that a purine-rich stretch would suffice for priming with oligo(dT). Dr. Wimmer mentioned that sufficiently high enzyme and primer concentrations resulted in nonspecific priming and gave examples.

Dr. Dalrymple querried Dr. Padmanabhan on the methodology of the direct enzymatic RNA sequencing he had presented which indicated that his DEN 2 RNA possessed a poly(A) tail and suggested that if only one of the base-specific nucleases were active it would appear that the RNA was polyadenylated. Dr. Padmanabhan was sure that his enzymatic procedures were correct but acknowledged that he did not include a known RNA as a control. Further questioning developed along the lines that since flavivirus RNA was hard to label enzymatically, a small contamination of the dengue RNA preparation with poly(A)-containing RNA might give the observed result. Dr. Padmanabhan replied that after end labeling, the RNA was size selected to be 8 kb or larger, and he felt confident that he was in fact labeling the dengue RNA.

Dr. Rice had shown a computer generated hydrogen bonded secondary structure which included the 3' terminus of YF virus in his presentation, and suggested that when the terminus was tied up in this structure it was difficult to modify. Dr. Brinton showed a similar structure for the 3' terminus of West Nile virus strain E101. Her sequence differs in only one out of the first 12 nucleotides from that published for another strain of West Nile by the Wenglers. The 3' terminus of West Nile is not included in this structure, however, and it is perhaps significant that she had no difficulty in labeling the 3' end with pCp. She had difficulty in using a primer made to the 3' end for cDNA transcription, however. This primer, made originally for use with JE virus RNA, would be bound within the region of secondary structure at the 3' end, and it was suggested that denaturation, perhaps with methyl mercury hydroxide, might be required as had been the experience of Dr. Fournier with JE virus.

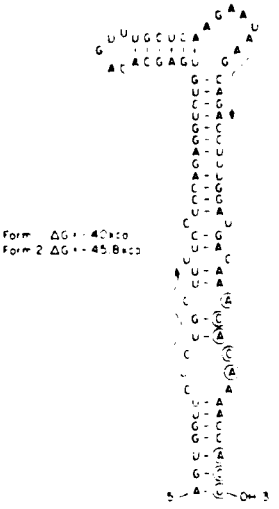
Dr. Westaway introduced his model for flavivirus replication based on UV inactivation of translation which requires internal initiation in order to interpret the results. This seems to conflict with the finding of a single open reading frame in the sequence of the yellow fever genome. Dr. Westaway cited polio virus RNA as an existing example of internal initiation. Dr. Wimmer countered that the evidence for internal initiation of translation in polio was from *in vitro* systems which he did not feel accurately reflected the *in vivo* conditions, the results being subject to variation in ionic conditions, Mg^{++} concentration, etc.



terminus is indicated by a dashed line above) during virus maturation and release. Proposed signal sequences for the cotranslational membrane insertion of GP19 and E, respectively, are located in the N- and C-terminal domains of GP19.

The extreme 5'- and 3'-terminal sequences of the genomic RNA are homologous to those found for another flavivirus, West Nile virus, and the complement of the 5'-terminal sequence (equivalent to the 3' terminus of the (-) strand) is related to the 3'-terminal sequence of the (+) strand. This suggests that the viral replicase/transcriptase may have related recognition sites for (-) and (+) strand synthesis. In addition, the 3' untranslated region contains three tandemly repeated sequences.

A stable secondary structure can be predicted for the 3'-terminal 87 nucleotides of the YF genome which, if conserved among flaviviruses, may be involved in RNA replication and could explain the observation that some flavivirus genomic RNAs are poor substrates for T4 RNA ligase and E. coli poly(A) polymerase.



As an extension of these studies on YF, we have begun to study Murray Valley encephalitis virus (MVE). MVE is found in Australia and New Guinea, and is thus geographically distinct from YF. In addition, YF and MVE differ in tissue tropism and pathogenesis, and show no cross-neutralization. Using genomic RNA from purified MVE, cDNA clones have been constructed and aligned with respect to each other by restriction enzyme mapping and nucleotide sequence analysis. After translation of MVE nucleic acid sequences into all six possible reading frames, they could usually be unambiguously aligned with the YF polyprotein sequence by dot matrix homology routines. From these preliminary data the following conclusions can be made:

1) MVE and YF show strong homology (~50% at the amino acid level) in the nonstructural region.

2) Much less colinear homology is found in the C protein where apparent translocations have taken place.

3) Two highly conserved nucleotide sequences are found immediately upstream from the 3'-terminal secondary structure proposed for YF.

In conclusion, besides providing insights into flavivirus genome structure and expression, the YF database should prove useful for rapid alignment and interpretation of other flavivirus sequence data as it becomes available. In addition, computer searches thus far have revealed no convincing amino acid homology between YF and any of the alphaviruses (or related plant viruses) which certainly justifies the reclassification of flaviviruses as a separate family.

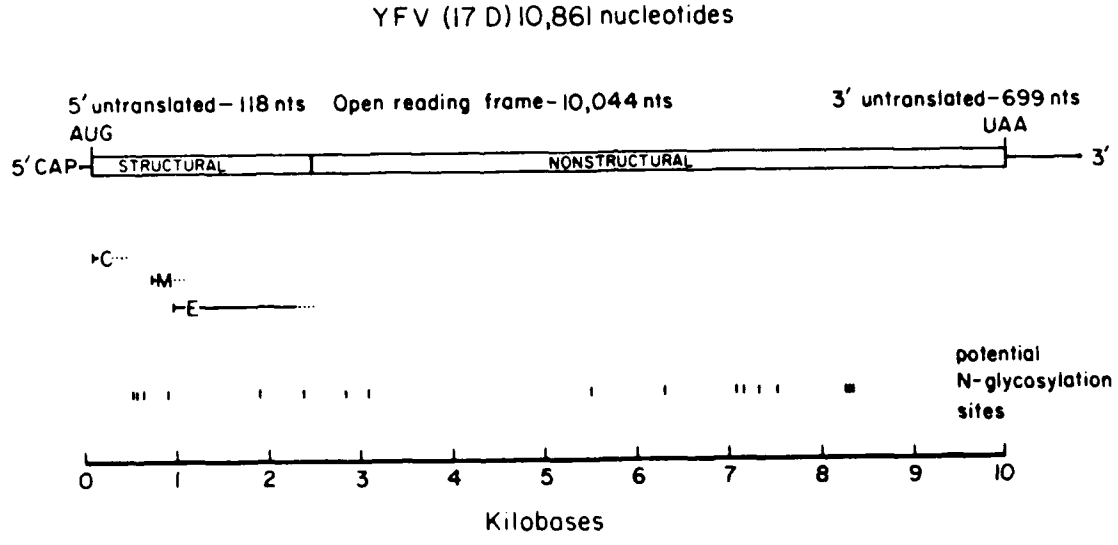
TITLE: Structural analysis of flavivirus genomes.

AUTHORS: Charles M. Rice,[1] Edith M. Lenches,[1] Lynn Dalgarno,[1,2] Sean R. Eddy,[1] Se Jung Shin,[1] Rebecca L. Sheets,[1,3] Dennis W. Trent,[4] and James H. Strauss[1]

ADDRESS: [1]Division of Biology, California Institute of Technology, Pasadena, CA 91125

TELEPHONE: (818) 356-4978

In order to study flavivirus genome structure and expression, cDNA clones have been constructed which together contain the entire nucleotide sequence of the yellow fever virus genome (YF, 17 D vaccine strain). Clones ranging from 2-7 kilobases in length were aligned by restriction analysis and sequenced using the base-specific chemical cleavage method. Inspection of this sequence reveals a single long open reading frame of 10062 nucleotides, which could encode a polyprotein of 3348 amino acids beginning from the first methionine residue. Comparison of this sequence with amino-terminal protein sequence data shows that the structural proteins are the first products encoded in this reading frame in the order 5'-C-M-E-3' (see below).



The C protein contains ~25% basic amino acid residues and a short uncharged region from residues 43-58. The N-terminal portion of the M protein is highly charged whereas the C-terminal domain is hydrophobic containing only a single charged residue (see Kyte and Doolittle hydrophobicity analysis below; search length = seven amino acid residues). The virion envelope glycoprotein (E) contains two potential N-linked glycosylation sites (denoted by asterisks) and a hydrophobic domain near the C terminus. Since all three mature structural proteins are encoded in the single long open reading frame and do not contain N-terminal methionine residues they must be produced by post-translational cleavage. Although intracellular forms of C and E have been identified, the M protein has not been detected in infected cells and may be produced by the late cleavage of glycosylated precursor GP19 (whose putative N

[2]Biochemistry Department, The Faculties, Australian National University, P.O. Box 4 Canberra, Australia 2600
[3]Department of Cellular and Molecular Biology, University of Utah, University Medical Center, Salt Lake City, UT 84132
[4]Division of Vector-Borne Viral Diseases, Center for Infectious Diseases, Centers for Disease Control, Fort Collins, CO 80522

TITLE: Synthesis and Cloning of cDNA from the Japanese Encephalitis Virus Genome

AUTHORS: P. C. McAda[1], T. L. Mason[1], C. S. Schmaljohn[2], J. M. Dalrymple[2] and M. J. Fournier[1]

ADDRESS: Department of Biochemistry, University of Massachusetts, Amherst, MA 01002[1] and Department of Viral Biology, U. S. Army Medical Research Institute of Infectious Diseases, Frederick, MD 21701[2]

TELEPHONE: (413)-545-2732/0353[1]; (301)-663-7241[2]

The genome of the Japanese encephalitis virus (JEV: Nakayama strain) is being cloned and characterized by sequence and expression analyses. The aims of the program are to conduct detailed gene structure-function studies, to acquire useful probes for diagnostic applications, and to explore the potential for developing an effective peptide-based synthetic vaccine. Thus far, 9 kilobases (kb) of the approximately 12 kb RNA genome have been cloned as cDNA in the bacterial plasmid, pBR322. Reverse transcription of the plus-stranded RNA was initiated from a synthetic oligonucleotide primer complementary to the 3'-terminus. The yield and quality of the cDNA synthesized were both markedly improved when the RNA was pre-treated with the denaturing agent methylmercury hydroxide and transcription was carried out in the presence of the commercial RNase inhibitor, RNasin. The first strand cDNA products ranged from 1-12 kb, with a substantial proportion corresponding to genomic length transcripts--possibly 50% of the product in one preparation.Under the conditions employed, no detectable synthesis was observed in the absence of primer.

The cDNA products have been cloned either as RNA:cDNA hybrids or as double stranded cDNA, the latter generated by self-primed second strand synthesis. The viral and plasmid sequences were joined for transformation through complementing poly dC, dG tails. The recombinant plasmids derived from the transformants contained cDNA inserts up to about 6 kb in size. When the cDNA was size fractionated prior to cloning, about 75% of the hybrid plasmids contained inserts larger than 1 kb and over 10% were in excess of 3 kb.

Cross-hybridization and restriction analyses were used to detect and order the overlapping cDNAs. Hybridization with full-length viral RNA served to verify that the resulting bank corresponds to cloned JEV sequences. As presently defined, the cDNA bank includes at least 9 kb of unique DNA, accounting for a minimum of 75% of the genome. The entire cloned region is contained in four overlapping fragments.

Sequence information obtained thus far for a 3'-proximal 1.4 kb segment revealed the occurrence of several translational open reading frame sequences (ORF). Most are too small to be significant but three elements could encode proteins of 8 to 16 kilodaltons. The presence of stop codons in all three reading frames for both strands indicates that this portion of the genome does not constitute a single, continuous ORF. Results from *in vivo* expression studies have verified the presence of ORF sequences, but it is not yet known whether any of these correspond to actual viral protein coding regions. A functional map of the genome will be developed by subcloning random cDNA segments into bacterial expression vectors and screening with immunological probes.

and pVV9 having about 1.4 kb and 1.65 kb cDNA inserts, respectively hybridized to each other and were found to share about 300 bp. The three clones, pVV1, pVV9 and pVV17 represent about 4.7 kb of DEN-2 viral genome.

We determined the complete sequence analysis of the cDNA insert in pVV17 by using the strategy described by Guo et. al. (Nucleic Acids Res. 11, 5521-5540, 1983) and chemical method of Maxam and Gilbert. This cDNA clone is 1971 nucleotides long. Translation of the DNA sequence into the amino acid sequence in all three reading frames of both DNA strands using a computer program revealed the presence of an open reading frame (ORF) coding for a polypeptide containing 621 amino acids.

We were interested in investigating whether these cDNA clones would be useful as type-specific hybridization probes. The clone pVV17 was radio-labeled by nick-translation and was used as probe for hybridization with viral RNAs isolated from Dengue virus types 1-4, Japanese Encephalitis (JE), GDVII (a mouse virus) Semliki Forest Virus (SFV), Western Equine Encephalitis (WEE), St. Louis Encephalitis (SLE), West Nile Encephalitis (WNE) and Kunjun. All viruses were grown in suckling mouse brain or mouse cells in tissue culture. We found that the clone pVV17 hybridized very specifically to Dengue virus type 2 RNA.

Structural analysis of two other cDNA clones pVV1 and pVV9 as well as the cloning of the remaining portion of the viral genome are in progress. This project was supported by U.S. Army Medical Research and Development Command Contract DAMD17-82-C-2051.

TITLE: Structural Analysis of Dengue Virus Type 2 Genome

AUTHORS: Vakharia, V., Yaegashi, T., Feighny, R., Kohlekar, S. and Padmanabhan, R.

ADDRESS: Dept. of Biochemistry, University of Kansas Medical Center, Kansas City, KS 66103 and Dept. of Virus Diseases, Walter Reed Army Inst. of Research, Wash. D.C. 20307

TELEPHONE: (913) 588-7018 and (202) 576-3012

The native Dengue virus type 2 RNA was found to be a poor template for oligo d(T)-primed cDNA synthesis. However, when the viral RNA was denatured by treatment with 10mM methylmercuric hydroxide, it served as a good template for cDNA synthesis suggesting that poly(A) tail was not accessible in the native RNA for reverse-transcriptase-catalyzed cDNA synthesis presumably due to the presence of secondary structure. We wanted to confirm the presence of poly(A) tail at the 3' end of viral RNA by direct sequence analysis. Viral RNA was labeled with ^{32}pCp catalyzed by RNA ligase and the 3' labeled RNA was subjected to partial cleavage by base-specific ribonucleases. Only ribonucleases Phy M (A + U specific) and U_2 (A specific) were able to generate a ladder of 3' labeled nucleotides, whereas RNase from B.cereus (C + U specific) RNase T_1 (G-specific) were not, confirming the presence of poly(A) tail at the 3' end of DEN-2 RNA.

For cDNA synthesis, DEN-2 RNA, denatured with methylmercuric hydroxide was used as a template for oligo d(T) primed cDNA synthesis. The first strand synthesized was estimated to be approximately 5 kilobases in length. For second strand synthesis, we used the approach described by Gubler and Hoffman (Gene 25, 266-269, 1983). The synthesis was catalyzed by the concerted actions of three enzyme activities, E.coli RNase H, E.coli DNA polymerase I and E.coli DNA ligase. Subsequent to oligo d(C) tailing, double-stranded cDNA was cloned at the PstI site in the polylinker (lac Z' coding) region of pUC13-1 vector. The recombinants were screened on agar plates containing IPTG, ampicillin and the chromogenic substrate for β galactosidase, x-gal, as well as by colony hybridization using labeled cDNA probe. There were a total of eleven recombinants containing various sizes of the cDNA inserts ranging from 1.95 kb to 0.95 kb. The cDNA clone, pVV17, having the longest insert (about 1.95 kb) was chosen for further characterization. The clones pVV2, 4, 7, 11 and 15 all hybridized to pVV17 and hence shared at least a portion of their sequences, whereas pVV1 and pVV9 did not. However, pVV1

one serotype occurs (4,5). A one-dimensional fingerprinting system has been used to determine the genetic variation within the dengue-1 serotype. Four dengue-1 virus isolates from Malaysia (provided by Dr T. Pang) and three dengue-1 isolates from Australia (provided by Dr B. Kay) were examined by digesting cDNA synthesized from the viral RNA with the restriction enzyme *Hae III*. The one-dimensional patterns obtained by polyacrylamide gel electrophoresis of the resulting fragments showed that the Malaysian isolates were all different (by at least one band) while the Australian isolates were identical. It was clear from these one-dimensional patterns that the 1981/82 isolates from Australia were not imported from Malaysia since the *Hae III* patterns were very distinct between the Malaysian and Australian isolates. Some of the *Hae III* fragments were shared by all 1981/82 isolates as well as the 1944 dengue-1 prototype virus.

In order to determine the molecular variation of some of these isolates in more detail, nucleotide sequencing using cloned dengue-1 specific DNA primers is underway.

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TITLE: Genetic Variation Among and Within Dengue Virus Serotypes

AUTHORS: Jan Blok

ADDRESS: Queensland Institute of Medical Research,
Bramston Terrace, Herston, Brisbane, Queensland, 4006, AUSTRALIA

TELEPHONE: (07) 529222

In order to determine the broad genetic relationship of the four dengue virus serotypes, cDNA-RNA hybridizations were carried out. Dengue viruses contain a single-stranded RNA genome of about 12,000 nucleotides. Variable amounts of near full-length cDNA were synthesized *in vitro* from 1 µg of RNA genome extracted from the prototype strains of the four dengue virus serotypes using an oligo(dT) primer and the enzyme, reverse transcriptase.

Published sequence data from the flaviviruses West Nile and Japanese encephalitis indicate that these RNA genomes do not contain a poly(A) tail (1,2). The nucleotide sequence data from yellow fever virus presented at the International Congress of Virology in Sendai, Japan (1984) by Dr C.M. Rice, showed that there is an adenosine rich region near the 3' end of the RNA genome. If dengue virus RNAs contain a similar region then it is conceivable that an oligo(dT) primer could bind to this region with variable efficiency.

By optimizing the *in vitro* cDNA synthesis conditions, enough dengue-1, -2, -3 and -4 specific cDNA probes were produced to study the genetic relatedness of all four dengue virus serotypes by cross cDNA-RNA hybridizations. These studies reveal that:

(a) the background of S_1 nuclease resistant hybrids formed with C6/36 cellular nucleic acid is about 10%,

(b) dengue-1 and dengue-4 produce hybrids which are about 70% resistant to S_1 nuclease,

(c) dengue-3 and dengue-4 RNA genomes show about 50% sequence homology,

(d) dengue-2 does not seem to be very closely related to any other dengue virus serotype,

(e) no difference can be detected between strains of the same serotype by this technique (3),

(f) dengue-2 cDNA does hybridize with the Australian flavivirus Edge Hill RNA (about 70%) and this relationship has also be detected by a dengue-specific monoclonal antibody (3).

The close genetic relationship between serotypes 1 and 4, 3 and 4 and the lack of a close relationship of serotype 2 with any other dengue virus serotypes suggest that the dengue viruses may differ in their evolutionary patterns.

Variation within one serotype cannot be detected by cDNA-RNA hybridization but a more sensitive technique such as ribonuclease T_1 oligonucleotide fingerprinting can be used. Oligonucleotide fingerprints of dengue-2 viruses have shown that variation within

TITLE: Sequencing of the St. Louis encephalitis virus genome

AUTHORS: A. Vance Vorndam, Dennis W. Trent, and Kotaro Yasui

ADDRESS: Centers for Disease Control, P.O. Box 2087, Fort Collins, CO 80522-2087

TELEPHONE: 303-221-6463

Cloning and sequencing of St. Louis encephalitis (SLE) virus, MSI-7 strain, has been initiated in our laboratory. MSI-7 is a "virulent" SLE strain isolated under epidemic conditions in 1975. Two sequencing procedures, primer extension on an RNA template and molecular cloning, are being employed. Primer extension sequencing (PES) is most useful for rapid scanning of specific genome regions and for linking cloned genome fragments. PES by the dideoxy method was begun by using primers to the 3' terminus of the genome and to the amino terminus of the glycoprotein gene. Initial results indicate that sequences near the 3' end of the genome are rich in G and may be involved in tertiary structure.

We have synthesized cDNA to the SLE genome using these two primers as well as calf thymus DNA. cDNA was synthesized with reverse transcriptase for the first strand and ribonuclease H/Polymerase I for the second strand. The fragments were cloned into E. coli K12 TB-1 using the pUC-18 plasmid. Clones primed with the glycoprotein primer are 900 to 1000 bases long and may contain both the M and C genes. Clones primed with the 3' primer or calf thymus DNA range from 500 to 3500 bases in length. Of the 40 clones now being worked with, 6 hybridize with the glycoprotein primer and 6 with the 3' primer. Preliminary results with restriction endonuclease mapping indicate that at least half of the SLE genome is covered by these clones.

I. Flavivirus Replication Strategy

TITLE: The Antigenic Structure of Japanese Encephalitis Virus Glycoprotein V3(E)

AUTHORS: Kotaro Yasui

ADDRESS: Department of Microbiology, Tokyo Metropolitan Institute for Neurosciences, 2-6 Musashidai, Fuchu, Tokyo, Japan
TELEPHONE: 0423(25)3881

Japanese encephalitis (JE) virus contains three structual proteins, V1(M), V2(C) and V3(E). It has been known by our studies and others that the major glycoprotein V3(E) has important role for flavivirus infection and antigenic determinant sites against neutralizing antibodies and also contains at least three antigenic determinants, flavivirus cross-reactive, subgroup reactive and viral species specific. These determinants seem to be correlated with the important biological properties, hemagglutination (HA) and neutralization of the viruses. It is important to elucidate the correlation between antigenic determinant sites of V3 protein and the biological activities for clarification of host defence mechanisms and development of potential vaccine. We analysed the antigenic structure of V3(E) protein by using monoclonal antibodies.

We used 18 monoclonal antibodies against V3(E) protein for the analysis and grouped them. To characterize the monoclonal antibodies and analyse the topography of the epitopes on V3(E) to which the monoclonal antibodies reacted, ELISA, HA inhibition (HI) and neutralization test and a competitive binding assay were performed. We could identify 8 distinct domains of antigenic determinants on V3(E) protein and grouped the antibodies.

Group 1 antibodies had high HI titre but did not show any neutralizing activity against JE virus. These antibodies showed high cross-reactivity with other flaviviruses by ELISA and HI but did not show any reactivity against alphaviruses. ELISA and HI titres of group 1 antibodies against the flaviviruses were similar to those against JE virus. It is clear that these antibodies are flavivirus cross-reactive HI antibodies. It is interesting that the antibodies could neutralize Dengue (D) 2, St. Louis encephalitis (SLE) and West Nile (WN) viruses but could not neutralize Murray Valley encephalitis (MVE) virus.

Group 2 antibodies had high HI titre and no neutralizing activity against JE virus and cross-reacted with same subgroup flaviviruses as JE virus, WN subset, but did not show any reactivity with D viruses. In this group the antibody cross-reacted with MVE virus, WN virus and SLE virus and the antibody cross-reacted only with MVE virus were included. Group 2 antibodies were therefore assigned subgroup cross reactive HI antibody.

Group 3 antibodies showed neutralizing activity but no HI activity and reacted only with JE virus. The antibody in this group strongly inhibited hemolysis and cell fusion causing by JE virus. In this group the strain specific antibody was included.

Group 4 and 5 antibodies showed neutralizing activity but no HI activity and reacted with same sub-group viruses as JE virus. In these groups the antibodies cross-reacted with MVE virus and WN virus and the antibodies cross-reacted with all the three viruses were included. There were no significant differences in antibody characteristics between group 4

and 5, however, distinct topographical difference in antigenic determinant sites were observed.

Group 6 antibody did not show any neutralizing and HI activity. The antibody reacted with MVE virus and WN virus too.

Group 7 antibodies showed both HI and neutralizing activity. In this group the antibodies cross-reacted only with MVE virus and the antibody cross-reacted both with MVE virus and WN virus were included.

Group 8 antibody showed high neutralizing activity but no HI activity and the reactivity was restricted only JE virus. This monoclonal antibody might be an authentic neutralizing antibody against critical site of the virus for neutralization because neutralizing titre of the antibody was nearly equal to ELISA titre, for example 10^5 neutralizing titre/$3x10^5$ ELISA titre.

Epitopes against neutralizing antibodies were sited relatively close each other, especially between the epitopes against group 3 and 8 antibodies, and far from a epitope of flavivirus cross-reactive HI antibodies. It became clear that important epitopes for viral infection, namely against neutralizing antibodies, were sited on restricted part of V3(E) and had relatively virus species specificity. These results indicate that it is possible to develop a synthetic vaccine against JE virus.

There were many highly cross-reactive epitopes on MVE virus except group 3 and 8 and the biological activities of antibodies against these cross-reactive epitopes shown on JE virus were also same on MVE virus, so there might be a great resemblance in antigenic structure of V3(E) protein between JE virus and MVE virus in WN subset viruses.

We also obtained strain specific monoclonal antibodies against V3(E), representatively clone 401 and N13, and attempted to group 11 JE virus strains known their sero-type, JaGAr-01 or Nakayama-NIH, by the neutralizing test using polyclonal hyper-immune sera with these monoclonal antibodies. Reactivity of monoclonal antibody 401 was restricted to JaGAr-01 type strains and N13 reacted only with Nakayama-NIH type strains. So these antibodies with another JE virus specific monoclonal antibodies are of practical use for rapid estimation of the virus species and type of strain. These epitopes against the strain specific antibodies were sited near the group 8 domain.

The mixture of cross reactive antibodies against different domains showed a considerably higher neutralization titres than the titres expected by simple dilution of neutralizing antibodies. These mixing effects were observed not only on JE virus but also against other WN subset viruses and may be reflected in cross neutralization among flaviviruses.

These result indicates that there are separately functional domains on JE virus V3(E) glycoprotein.

TITLE: Mapping the Antigenic Determinants of Japanese Encephalitis Virus: Strategy and Progress Report

AUTHORS: P. W. Mason[1], P. C. McAda[1], T. L. Mason[1], M. J. Fournier[1], M. K. Gentry[2], J. M. Dalrymple[3] and C. S. Schmaljohn[3]

ADDRESS: Department of Biochemistry, University of Massachusetts, Amherst, MA 01002[1], Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307[2] and Department of Viral Biology, U. S. Army Medical Research Institute of Infectious Diseases, Frederick, MD 21701[3]

TELEPHONE: 413-545-3122/0353[1], 202-576-3527[2] and 301-663-7241[3]

Monoclonal antibodies to the major antigens of Japanese encephalitis virus have been used to distinguish topographically and functionally different epitopes on the V3 envelope glycoprotein (1,2). One striking observation was that passive administration of certain antibodies could protect mice from a lethal challenge of JEV while other, non-protective antibodies actually resulted in increased mortality and decreased survival time of mice challenged with sublethal doses of the virus (2). These results underscore the importance of mapping the antigenic determinants to specific sites on the V3 protein sequence and establishing the molecular basis for the different functional properties of these epitopes.

Our strategy for epitope mapping is based on plasmid and phage λ expression vectors that allow the construction of active hybrid genes between open-reading-frame (ORF) DNA and vector-encoded E. coli β-galactosidase (lacZ). The plasmid vectors are constructed so that insertion of an ORF sequence in the correct frame restores translation of the downstream lacZ region to yield functional β-galactosidase (3,4). Thus, ORF sequences can be identified by plating transformants on lacZ indicator plates. In the phage vector, λgtll, the in-frame insertion of cDNA segments is into the distal portion of lacZ resulting in an inactive lacZ fusion protein (5). Although each expression system offers unique advantages, we are convinced that the λgtll vector provides the best method for the identification of the viral epitopes corresponding to exisiting immunological probes (cf. 6). Since available monoclonal antibodies and hyperimmune ascitic fluids have combined reactivities with polypeptides that account for over 80% of the coding capacity of the JEV genome, we are developing banks of JEV cDNA (7) in λgtll for expression analysis.

Methods have been optimized for the random fragmentation of cloned JEV sequences and for their efficient insertion into λgtll. So far, we have immunologically screened 6,000 recombinants from a bank constructed with 70 bp ($\bar{M}_n$) fragments and have not been able to detect the expression of any JEV epitopes. We are currently constructing banks with 1-2 kb and >2 kb fragments with the expectation that many epitopes will be more effectively presented in longer polypeptide fragments.

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TITLE: Functional and Immunochemical Characterization of Antigenic Determinants on the Tick-Borne Encephalitis Virus Glycoprotein.

AUTHORS: F.X.Heinz, R.Berger, G.Winkler, W.Tuma, C.Mandl, C.Kunz.

ADDRESS: Institute of Virology, University of Vienna, Kinderspitalgasse 15, A-1095 Vienna, Austria.

TELEPHONE: (222) 43 15 95.

By the use of monoclonal antibodies we have established a model showing the serological specificities, functional activities and spatial relationships of eight distinct epitopes on the TBE virus glycoprotein. Based on mutual blocking of monoclonal antibodies in competitive binding studies most of these epitopes cluster to form two major antigenic domains (A and B). Both domains are inhomogeneous with respect to serological specificity and contain epitopes which differ with respect to their involvement in hemagglutination inhibition, neutralization and passive protection. These marked differences in functional activities are not only a matter of antibody avidity, since we have shown that certain nonneutralizing antibodies have higher avidities than neutralizing antibodies binding to closely adjacent epitopes. These findings are especially relevant to the design of synthetic peptides for immunization, since they reveal the importance of the precise selection of sequences involved in functional activities.

Simultaneous binding studies of antibody pairs for the analysis of overlapping epitopes revealed that antibodies may not only block each other but in contrast may even enhance the binding of other antibodies. This leads to a complex network of interactions between antibodies to distinct nonoverlapping epitopes which can either be unidirectional or bidirectional. The quantitative evaluation of binding data by Scatchard analysis revealed that the observed phenomenon is due to an up to six-fold increase of antibody avidity. In the present system enhancement of antibody binding is not dependent on antibody bivalency since it could also be demonstrated with purified Fab fragments acting either as enhanced or as enhancing antibody. It can therefore be assumed that binding of antibodies to certain epitopes on the TBE virus glycoprotein induces conformational changes in distant parts of the molecule which can result in increased avidity of antibodies directed to conformationally changed epitopes.

The structural properties of each mononclonal antibody-defined epitope were assessed by analyzing the influence of conformational changes on the antigenic reactivity. All epitopes of domain A were extremely denaturation-sensitive and lost their activities upon incubation at pH 5.0 and by treatment with guanidine-HCl/urea, SDS or reduction and carboxymethylation. The second major domain B on the other hand proved to be denaturation-resistant and was destroyed only by reduction and carboxymethylation. A very similar picture was obtained upon proteolysis using trypsin, α-chymotrypsin and thermolysin, epitopes of domain A being sensitive and those of domain B being resistant. Each of the proteases used yielded fragments of mol.weight approximately 9000 which were stable to further proteolysis and were shown by immunoblotting to carry all the epitopes of domain B. Upon immunization of mice, these fragments induced glycoprotein-reactive antibodies which were also functionally active in hemagglutination inhibition and neutralization.

Amino acid analysis of these fragments after reversed phase HPLC and identification by dot immunoassays revealed the presence of potential additional cleavage sites for each of the proteases used suggesting the presence of a strongly stabilized core structure which is resistant to further proteolysis. It was also shown that after reduction of disulfide bridges this domain has a strong tendency to renaturation and there is evidence that the antigenic reactivity of epitopes of domain B is dependent on a core structure which is stabilized by disulfide bridges. Although epitopes of domain A are much more sensitive to denaturation, both domains seem to be strongly dependent on the native conformation of the molecule.

TITLE: Protection Against 17D Yellow Fever Virus (17D) Encephalitis in Mice by Passive Transfer with Monoclonal Antibodies and by Active Immunization with a Purified 17D Nonstructural Glycoprotein gp48 (NV3)

AUTHORS: Jacob J. Schlesinger, Michael W. Brandriss and Edward E. Walsh

ADDRESS: Rochester General Hospital, University of Rochester
1425 Portland Avenue, Rochester, NY 14621

TELEPHONE: 716 338-4092

High-titered neutralizing activity against Togaviruses has, in general, been a property of type or strain-specific monoclonal antibodies (MAB) which are usually most effective in passive protection models in preventing lethal encephalitis after challenge with homologous virus. However, more broadly reactive MAB with little or no neutralizing activity have been shown to protect against challenge with heterologous as well as homologous virus. Among alphaviruses this property has been correlated either with high avidity (Venezuelan equine encephalitis)(1) or the ability to lyse virus-infected cells in vitro in the presence of complement (Sindbis)(2). Among flaviviruses only neutralizing MAB protected against challenge with tick-borne encephalitis (3) and solid protection against St. Louis encephalitis (SLE) was conferred only by a type-specific high-titered neutralizing MAB (4) suggesting that the development of a vaccine utilizing flavivirus cross-reactive antigenic epitopes is probably not feasible since they appear to be much less protective than type-specific ones. These studies have all utilized MAB to virion envelope proteins; a possible protective role of antibodies to nonstructural proteins has not been previously investigated.

MAB produced to vaccine strain 17D have been described previously (5,6). Thirteen of the nineteen were IgG MAB directed to the envelope protein (E). These were categorized into several groups (Table 1) on the basis of their serologic activity which included neutralization by a conventional plaque reduction method (PRNT) in Vero cells, HAI, complement fixation (CF), and immunofluorescent assays. The only IgM MAB (8A3) produced was strain-specific for 17D and neutralized it in high titer. At least nine epitopes on E

Table 1. PROTECTION AGAINST INTRACEREBRAL CHALLENGE WITH 17D BY MAB TO E (V3)

			Flavivirus	N (Vero)		Protection
17D	Group	Designation	Specificity	17D	Asibi	
Antibodies	A	8A3	Strain	+	-	-
	B	4E8,2C9,2E10	Type	+	+	+
	C	2B8,5E3,2D12,3A3,4E1,5E5	Type	-	+	+
	D	4E11,5E6,5H3	Group	-	+	+
	E	3E9	Type	-	-	+
Dengue 2		4G2,1B8	Group	-	ND	+
Antibodies		3H5	Type	-	ND	-

have been identified by patterns of seroreactivity and results of competitive binding assays (7). All anti-17D MAB as well as three previously reported MAB to dengue 2 virus(DEN 2)(8) were employed in passive protection experiments using intracerebral challenge with 10^3 to 10^4 LD_{50} of 17D in Swiss (CD-1) and BALB/c mice. MAB in the form of immune ascites of known Ig concentrations (400,40,4,0.4 ug/mouse) were injected intraperitoneally 24 hrs before challenge. The IgM MAB (8A3) failed to provide any protection despite its high-titered neutralizing activity possibly because of inability to cross the blood-brain barrier. All 17D IgG MAB to E in high dose provided solid protection regardless of epitope specificity or ability to neutralize 17D in vitro. 17D MAB with high in-vitro neutralizing titers (Group B) were in general protective at lower dose (0.4-4 ug) than were nonneutralizing MAB (40-400 ug). However, two type-specific nonneutralizing MAB (4E1, 3E9) provided similar protection at low dose; this could not be attributed to high avidity. Two flavivirus group-reactive DEN 2 MAB also protected. One of these (4G2) was comparable to high-titered neutralizing MAB to 17D in conferring protection at low dose. MAB 3E9 and anti-DEN 2 4G2, which were nonneutralizing in Vero cell assay did exhibit neutralizing activity when assayed in mouse neuroblastoma cells providing a possible explanation

for their protective capacity. Recognition of conditional neutralization activity based on choice of cell type indicates that reliance on conventional plaque assays to define neutralizing capacity of MAB may be inadequate.

Two of five MAB obtained to the 17D-specified nonstructural protein gp48 (NV3) also protected against challenge. Similar to results previously reported by others with Sindbis virus (2), their capacity to protect correlated with their CF activity and their ability to lyse virus-infected cells in-vitro in the presence of complement as measured by a ^{51}Cr release assay (Table 2). Utilizing the same assay, complement-dependent cytolysis was not detected with any of the anti-E MAB.

Table 2. PROTECTION AGAINST INTRACEREBRAL CHALLENGE WITH 17D BY MAB TO gp48 (NV3)

Antibody[a]	Isotype	17D CF Titer	No. Survivors No. Challenged	% ^{51}Cr Release
17DIAF	-	1.8[b]	8/8	45
1A5	2a	4.2	15/18	44
8G4	2b	3.0	9/16	36
2G2	3	< 0.9	4/16	9
4E3	1	1.8	2/16	12
2D10	1	< 0.9	1/19	6
PC5	2a	ND	0/10	2
MOPC21	1	ND	0/16	8

[a] 400 ug monoclonal antibody or myeloma protein i.p [b] $-\log_{10}$

To examine the immunogenicity of gp48, the protein was purified by affinity chromatography using MAB 8G4 as the ligand. Purity was established by silver staining of the product after SDS-PAGE and by monospecificity of sera obtained from rabbits hyperimmunized with purified gp48. A group-reactive site was identified on 17D gp48 by binding of polyclonal antibodies raised against DEN, SLE and Powassan viruses. Results of challenge after intraperitoneal immunization with purified gp48, infectious 17D virion or control ovalbumin are shown in Table 3. Mice were bled the day before challenge and sera within each group pooled. Prechallenge sera from mice given gp48 were monospecific as determined by radioimmunoprecipitation. Immunization with two doses of gp48 with or without complete Freund's adjuvant(CFA) gave complete protection in the absence of neutralizing antibodies.

Table 3.PROTECTION AGAINST INTRACEREBRAL CHALLENGE WITH 17D BY INOCULATION WITH gp48(NV3)

Antigen (i.p.)[a]		Pre-challenge Serum Titer ($-\text{Log}_{10}$)		No. survivors No. Challenged
Prime	Boost	gp48 ELISA	PRNT	
17D virion	none	3	2.5	8/8
gp48/CFA	gp48	5	< 1.3	11/11
gp48	gp48	3	< 1.3	11/11
CFA	none	< 1	< 1.3	2/10
OA/CFA	OA	< 1	< 1.3	2/10
OA	OA	< 1	< 1.3	4/10[b]
none	none	< 1	< 1.3	0/11

[a] 17D 10^5 PFU; gp48 15 ug; OA 15 ug [b] 2/4 survivors paralyzed

The ability of flavivirus group-reactive antibodies to the E protein to provide protection against viral infection as well as the protection provided by immunization with gp48 which bears a group reactive determinant(s) may have implications in regard to flavivirus vaccine design.

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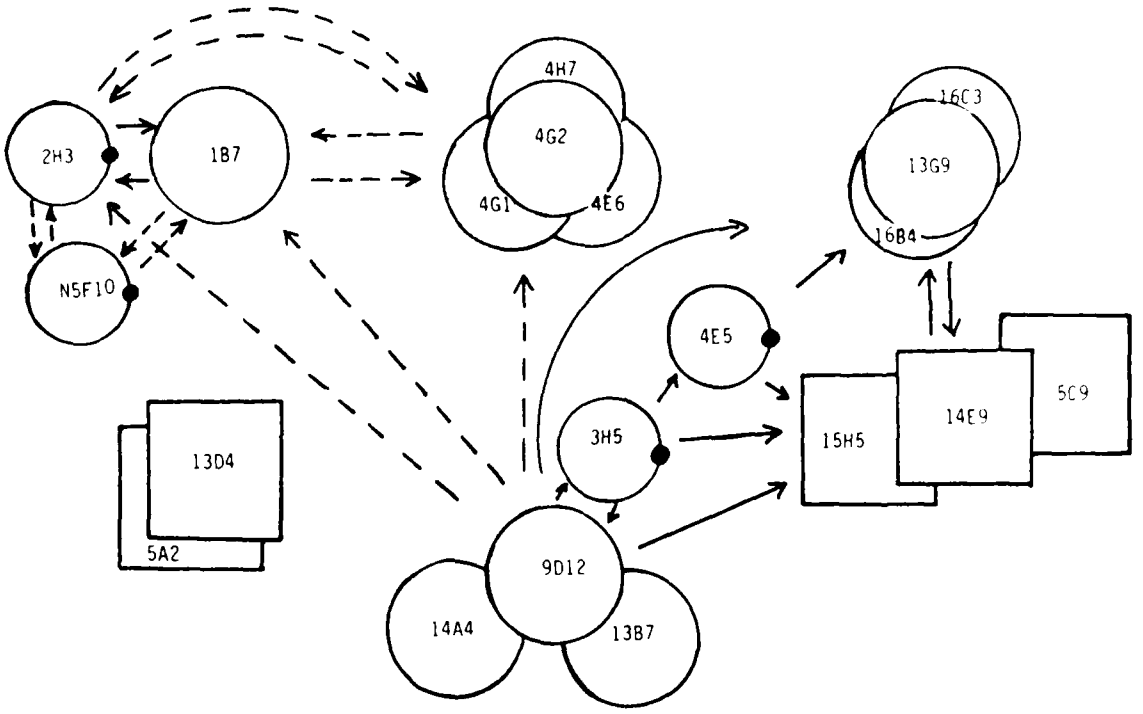
TITLE: DENGUE EPITOPE MAPPING: COMPARISON OF THE FOUR DENGUE SEROTYPES

AUTHORS: Donald S. Burke, Mary Kay Gentry, Erik A. Henchal, Robert J. Feighny, and Walter E. Brandt
ADDRESS: Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, D.C., 20307-5100
TELEPHONE: (202) 576-3757 or 3012

We sought to develop a large panel of dengue serotype-specific, dengue complex-reactive, and flavivirus group-reactive mouse monoclonal antibodies (MAB). Prototype strains of all four serotypes (DEN-1 Hawaii, DEN-2 New Guinea C, DEN-3 H87, and DEN-4 H241) were used to immunize BALB/c mice, and spleen cells from immunized mice were fused with P3x63Ag8 myeloma cells. Antibody-producing clones were identified by RIA screening of cell culture supernatant fluids for binding of immunoglobulin to lysates of homologous virus-infected LLC-MK2 cells. The binding spectrum of each positive MAB cell culture fluid (CCF) was determined by antibody capture solid phase RIA or ELISA using a battery of 8 flavivirus antigens (supernatant fluids of C6/36 Aedes albopictus mosquito cells infected with DEN-1, DEN-2, DEN-3, DEN-4, JE, TMU, or WSL, or LGT mouse brain antigen). The relative antigen binding activity (RABA) of each MAB CCF for each antigen was calculated as the antigen bound (CPM or OD) expressed as the per cent of a standard, strongly pan-group reactive MAB AD2.4G2. Clones with strong binding activity (RABA >40 to at least one antigen) were studied further. From each group of MAB CCF's which shared a common binding spectrum, one MAB was chosen for expansion (as ascitic fluid) and direct labelling with either I-125 or peroxidase. Low dilutions of CCF's were used to competitively block attachment of a limiting dilution of the labelled MAB. Identity was operationally defined when two MAB's had a common binding spectrum and at least one-way competitive binding could be demonstrated. When a CCF was encountered that did not block existing labelled MAF's with the same binding spectrum, that clone was in turn expanded, labelled, and used in competitive blocking assays. The cycle was repeated until the identities of all MAB's were established.

Of the 143 positive CCF's tested, 67 strongly bound at least one dengue serotype. Serotype-specific, complex-reactive, and group-reactive epitopes were identified; however, epitopes with varying subcomplex and subgroup cross-reaction patterns were also detected. All MAB's labelled were isotyped as either gamma-1/kappa or gamma-2a/kappa. Several factors were found to affect epitope accessibility: source of antigen (mosquito cell versus mouse brain); media conditions (pH); and concurrent binding of other monoclones at other epitopes (resulting in increased binding or "promotion"). Twenty-one unique but in some cases overlapping MAB's were found to react with DEN-2 (4 type, 4 subcomplex, 5 complex, 4 subgroup, and 4 group). Two distinct non-overlapping group-reactive epitopes were found to be present on all flaviviruses tested, and two distinct dengue complex epitopes were detected. Groupings of MAB's based on quantitative binding spectra (eg, DEN-1>DEN-2>DEN-3>DEN-4, or DEN-1 = DEN-2 = DEN-3 = DEN-4 predicted identity based on cross-blocking studies. Two monoclones, AD3.1B7 (subgroup-reactive) and AD2.4G2 (group-reactive), reciprocally promoted each other on all four serotypes. Only two other MAB's, AD2.2H3 and AD2.N5F10 (both DEN-2 specific) were strongly promotable. Binding at any one of at least four distinct other epitopes non-reciprocally promoted 1B7 and/or 4G2. MAB's with strong neutralizing activity were identified for DEN-1, DEN-2, and DEN-3, but none was found for DEN-4. Surprisingly, many of the strongly

neutralizing MAB's had subcomplex or subgroup binding spectra, although the homologous serotype was preferentially neutralized. A cartoon of the DEN-2 epitope map is shown below. Maps of the other dengue serotypes showed similar relationships between the group, subgroup, and complex-reactive epitopes. MAB's 15H5 and 5C9 immunoprecipitate a 20-22 kilodalton viral protein, while MAB's 2H3, 1B7, 4G2, 13D4, 9D12, and 3H5 immunoprecipitate a 60-62 kilodalton protein. The protein targets of MAB's 13G9, N5F10, and 4E5 are not yet resolved.



Large circle = flavivirus group or sub-group epitope; square = DEN complex epitope; small circle with dot = type specific epitope; dashed arrow = promotion; solid arrow = blocking. Overlaps are MAB's with similar binding spectra which cross block but have other minor but reproducible differences.

TITLE: Flavivirus Immunology. Round Table Discussion II

AUTHORS: James S. Porterfield and Walter E. Brandt

ADDRESS: Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, OX1 3RE. England, and Walter Reed Army Institute of Research Washington, D.C. 20307-5100

TELEPHONE: 0865 57321 (Oxford); 202 576-3626 (Washington, D.C.)

The session opened with a brief presentation by Carol Blair on polyacrylamide gels of radioimmune precipitates of Japanese encephalitis virus infected cell extracts. The virus-specific proteins that were precipitated exceeded the coding capacity of the genome, and this suggested some post-translational processing. Two dimensional peptide mapping revealed similarities between some of the larger and smaller peptides. Pulse chase experiments suggested some chasing of material from the larger to the smaller peptides. Generation of temperature-sensitive mutants has not resulted in the isolation of any processing mutants thus far.

Eckard Wimmer stressed the importance of configurational arrangements in epitope mapping. An antigenic site may consist of a single amino acid sequence that can fold in different configurations. Each configuration in turn may represent a separate epitope of that antigenic site. Some individuals believed that a single critical site or epitope is involved in virus neutralization, while others believed that virus neutralization was a more complex phenomenon. Donald Burke had shown with monoclonal antibodies that more than one epitope was involved in potent serotype-specific neutralization. Monoclonal antibodies directed against epitopes found on several different flaviviruses were also very effective in neutralization reactions. E. G. Westaway reminded the group that he had introduced the concept of "variable critical areas" in his anaylsis of the mechanisms of flavivirus neutralization.

Michael Brandriss questioned the practical use of the term non-neutralizing antibodies, pointing out that some monoclonal antibodies, while failing to produce neutralization of a virus in cell culture assays nevertheless conferred protection when the same virus-antibody mixtures were assayed in mice.

Gerald Cole was interested in the report on neutralization of heterologous viruses by monoclonal antibodies which failed to neutralize the homologous virus. Walter Brandt said that a number of people had obtained similar results, and perhaps they were the consequence of the stimulation of antibodies against a minor epitope in the immunizing virus reacting with the same epitope which was more accessible on a closely related virus for a neutralization reaction.

James Porterfield commented that the antigenic analysis of the flaviviridae could be likened to the complexity of the Salmonellae in which group-specific antigens and type-specific antigens contribute to the specificity of individual members. However, with flaviviruses, the group-specific epitopes were not necessarily the same chemical structures for all members of the family. There was evidence that monoclonal antibodies could detect cross reactions between different flaviviruses which were widely separated on the basis of serological tests with conventional polyclonal antibodies. He also stressed the need to use a wider panel of flaviviruses when categorizing monoclonal antibodies as type specific or cross reactive.

III. Future Vaccine Strategy

TITLE: CURRENTLY AVAILABLE VACCINES FOR JAPANESE ENCEPHALITIS, AND FUTURE RESEARCH

AUTHORS: Robert E. Shope

ADDRESS: Yale Arbovirus Research Unit, Box 3333, New Haven, CT 06510

TELEPHONE: 203/785-4821

There are currently available both inactivated and live attenuated vaccines for Japanese encephalitis. The inactivated vaccines have been used in Japan, China, Taiwan, and Korea for over two decades. The attenuated vaccines are still experimental for human use, but have been used extensively to immunize horses and swine.

Formalin inactivated vaccines produced in Japan, Taiwan, and Korea utilize the Nakayama strain grown in brains of mice 3 to 4 weeks of age. Basic myelin protein is removed by protamine and sucrose density centrifugation. These vaccines are safe and efficacious. Recent studies in Japan indicate that there are antigenic differences among Japanese encephalitis strains, the strains falling into two groups. A bivalent vaccine including both Nakayama and Beijin-1 strains protects mice against both groups of virus.

Formalin inactivated vaccine produced in China utilizes primary hamster kidney as substrate and human albumin as stabilizer. There are production facilities in 6 major regions of China. The facility in Shanghai alone produces approximately 40,000,000 doses per year.

Live attenuated Japanese encephalitis vaccines have been developed for human use in China by multiple passages in primary hamster kidney, and also by U-V mutagenesis, passage in mice and chick embryo culture, and clonal selection for avirulence for mice. There are at least 3 candidate vaccines, all derived from the SA 14 parent virus. Neutralizing antibody response in children to the attenuated viruses has been variable. The seroconversion rates were higher in both swine and human beings with vaccines of higher titer. There was 85 to 96 per cent protection in children receiving the 5-3 vaccine; antibody persisted over a year. The attenuated vaccines, where tested do not replicate well in mosquitoes, are small plaque, and not ts. A major impediment to their use is the difficulty maintaining the cold chain in China.

Future JE vaccine research is needed, both for improvement of conventional vaccines, and for development of new approaches. Comparative field trials of existing vaccines from different countries and additional studies of low-passage field strains for antigenic variation are needed. Improved adjuvants could markedly increase the efficacy of the inactivated vaccines. Almost nothing is yet known about the mechanism by which Japanese encephalitis viruses manifest attenuation.

New approaches include subunit vaccines produced by recombinant DNA technology, use of vaccinia as a vector, and as yet untried methods to deliver vaccine to amplifying hosts such as swine and birds.

TITLE: Modernization of Yellow Fever Vaccine

AUTHORS: Thomas P. Monath, M.D.

ADDRESS: *Division of Vector-Borne Viral Diseases, Centers for Disease Control, P.O. Box 2087, Fort Collins, CO 80522-2087

LEPHONE: 303-221-6400

Yellow fever 17D vaccine is still produced in embryonated chicken eggs according to methods established 40-45 years ago. Although the vaccine is extremely safe and highly effective, a number of problems exist, including: (1) thermal instability and short shelf life; (2) limited stockpiles and emergency production capability; (3) residual neurovirulence, limiting use in children under 1 year of age; (4) contamination of many products with avian leukosis virus; (5) relatively high cost; and (6) heterogeneity of vaccines in terms of substrain and passage history, RNA fingerprints, and plaque subpopulations.

Three strategies may be pursued for improvement. One of these, modernization of equipment and increased efficiency of production under present manufacturing methods, is already underway is some institutes. The second, development of a cell culture vaccine, has been repeatedly discussed by panels of experts at PAHO/WHO, and offers a number of advantages. Foremost among these are (1) the well-documented effective and long-lasting protective response induced by live attenuated 17D vaccine and (2) the possibility that virus yields in cell cultures will be up to 100 times those in eggs. The present availability of genetic markers, especially sequence analysis of the viral genome, and of monoclonal antibodies should simplify vaccine development and quality control. Studies of the molecular basis for and eventual elimination of genetic heterogeneity and neurotropism should probably be pursued as a separate area of research.

The third approach would harness biotechnological methods for the production of an antigenically defined subunit vaccine in an appropriate expression system or the incorporation of yellow fever viral genes in a vector, such as vaccinia. Given the rapid recent developments in our understanding of the gene organization of yellow fever 17D virus, it seems appropriate to pursue the feasibility of this approach to a third generation vaccine.

*Until October 1985: Gastroenterology Unit, Massachusetts General Hospital, Boston, MA 02114 (617) 726-3766

TITLE: Theoretical and practical considerations for dengue vaccination

UTHORS: Kenneth H. Eckels

DDRESS: Department of Biologics Research, Walter Reed Army Institute of Research, Washington, DC 20307-5100

EPHONE: (301) 427-5208

Each of the live-attenuated vaccine candidates for dengue (DEN) types 1, 2, and 4 re selected on the basis of laboratory and animal markers that were associated with tenuation. These included small plaque size, temperature sensitivity, reduced repli- tion in monocyte cultures, and reduced replication in mosquitoes, mice and monkeys. ch of the cloned vaccine strains were found to be different from non-attenuated parent ruses by each of these criteria. Although the three vaccine candidates were similar these criteria, the human response varied considerably. Clinical symptoms ranged om mild to severe and seroconversion was not predictable, especially in yellow fever n-immunes. These results indicated that the markers used for measuring attenuation re inadequate to predict the human response to vaccination. Additionally, the rhesus nkey was not a good animal model for dengue disease; clinical dengue fever or more vere forms of dengue are not reproducible in this animal.

In all three cases, the vaccine strains were produced after multiple passage and oning in mammalian cell cultures. The accumulation of genetic mutations during ssage resulted in a virus with reduced infectivity. Although some cell cultures allow troduction of mutations more predictably than others, there is no one "system" of ll culture passage that will yield, after a series of virus passages, a seed with all the characteristics required for human vaccination.

A more systematic approach to designing a DEN vaccine virus would be to first map e genome of the viral RNA to gain information on gene location and function. This uld be followed by site-directed mutagenesis in one or more genes. The resultant rus clones would then be screened by all of the laboratory and animal markers availa- le that could predict attenuation. The currently used markers, as noted above, are adequate for predicting a human vaccine response. Therefore new markers will have to developed or human testing must be accelerated if this can be combined with adequate fety.

Another approach to live DEN vaccination would be to use an already successful ve vaccine, such as the yellow fever 17D strain, that could be genetically manipulated contain DEN immunogenic epitopes. This would entail substituting all or part of the jor envelope glycoprotein of YF with that of DEN via recombinant techniques and taining a virus with the replication characteristics of YF and the antigenic specificity of DEN. Other viruses such as adenovirus and vaccinia could be used as cipients for DEN antigen genes as long as the attenuation characteristics of the cipient virus was not changed to any great degree and DEN antigens were expressed r stimulation of an immune response. Using these recombinant vaccines, optimal N immune responses may be obtained without having to be concerned with clinical ccine-related DEN disease.

For the development of non-infectious DEN vaccines, expression of immunogenic oteins in bacteria, yeast, or mammalian cells would be required as a first step. quencing and gene mapping are not absolutely required for this type of vaccine, but order to identify neutralization epitopes, and to synthesize these proteins, this uld become necessary. This class of vaccines would not require the same types of sts needed for live vaccines prior to human testing and the emphasis would be on monstrating an adequate immune response.

Finally, how will the new generation of vaccines fair in light of current and osed Federal regulations? In order to begin human clinical trials of these ucts, the FDA has to review data on the history, safety, production and efficacy he proposed vaccine. As in the past, a master cell bank that is fully characterized tested for adventitious agents is the cornerstone of production. Whether the cell acterial, yeast, or mammalian, the same criteria is applied. If an expression or is introduced into the cell, then it will be required that the vector be acterized as well as the vector-host cell combination. Expressed gene products be purified and assayed to demonstrate freedom from protein and nucleic acid amination using in vitro and animal assays. New Federal regulations are being osed so that IND submissions for new biologicals will be facilitated to expedite ical research studies.

Dr. Elizabeth Earley
Chief, Department of Cell Culture Production
Virology Division
US Army Medical Research Institute of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Kenneth H. Eckels
Assistant Chief
Department of Biologics Research
Division of Communicable Disease and Immunology
Walter Reed Army Institute of Research
SGRD-UWF-I
Washington, DC 20307-5100

Dr. Gerald A. Eddy
Consultant, Infectious Diseases
ImmuQuest Laboratories, Inc.
1600 East Gude Drive
Rockville, MD 20850

CPT Michael E. Faran
Virology Division
US Army Medical Research Institute of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Reiner Feick
Postdoctoral Research Associate
Department of Biochemistry
University of Massachusetts
Amherst, MA 01003

Dr. Bob Feighny
Microbiologist
Department of Virus Diseases
Walter Reed Army Institute of Research
SGRD-UWF-E
Washington, DC 20307-5100

Dr. Maurille J. Fournier, Jr.
Professor
Department of Biochemistry
University of Massachusetts
Amherst, MA 01003

Dr. George R. French
Government Services Division
The Salk Institute
P.O. Box 250
Swiftwater, PA 18370

Dr. Mary Kay Gentry
Division of Biochemistry
Walter Reed Army Institute of Research
Washington, DC 20307-5100

Dr. Barry Gorman
Queensland Institute of Medical Research
Bramston Terrace, Herston
Brisbane, Queensland 4006
Australia

COL Michael Groves
Director, Military Disease Hazards Research Program
US Army Medical Research and Development Command
Fort Detrick
Frederick, MD 21701

Dr. Janet Grun
Associate Professor
Wistar Institute
36th and Spruce Streets
Philadelphia, PA 19104

Dr. Scott B. Halstead
Associate Director
Health Sciences Division
The Rockefeller Foundation
1133 Avenue of the Americas
New York, NY 10036

eter G. Canonico
hief, Department of Antiviral
Studies
S Army Medical Research Institute
of Infectious Diseases
uilding 1425
ort Detrick
rederick, MD 21701

)r. Robert Chanock
.aboratory of Infectious Diseases
lational Institute of Allergy and
Infectious Diseases
)uilding 7, Room 301
lational Institutes of Health
)000 Rockville Pike
)ethesda, MD 20205

)r. Vina Churdboonchart
)epartment of Pathobiology
'aculty of Science
lahidol University
Rama 6 Road
)angkok 10400
Thailand

Gary G. Clark
JS Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Graham Cleaves
Department of Microbiology
University of Medicine and
Dentistry of New Jersey
Rutgers Medical School
P.O. Box 101
Piscataway, NJ 08854

Dr. Francis E. Cole, Jr.
Microbiologist
Virology Division
JS Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, Maryland 21701

Dr. Gerald A. Cole
Professor of Microbiology
Department of Microbiology
University of Maryland School
of Medicine
660 West Redwood Street
Baltimore, MD 21201

Dr. Marc S. Collett
Director of Molecular Biology
Molecular Genetics, Inc.
10320 Bren Road East
Minnetonka, MN 55343

Dr. Lynn Dalgarno
Professor, Australian National
University and the
Division of Biology
California Institute of Technology
Pasadena, CA 91125

Dr. Joel M. Dalrymple
Chief, Department of Viral Biology
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701-5011

COL Harry G. Dangerfield
Commander
US Army Medical Materiel
Development Agency
Fort Detrick
Frederick, MD 21701

Tom Dreier
Microbiologist
Pathology Division
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Natth Bhamarapravati
Professor of Pathology and
Rector, Mahidol University
Rector's Office
c/o Siriraj Hospital
Bangkok 10700
Thailand

Dr. Carol D. Blair
Associate Professor
Department of Microbiology
Colorado State University
Fort Collins, CO 80523

Dr. Jan Blok
Senior Virologist
Queensland Institute of
Medical Research
Bramston Terrace, Herston
Brisbane, Queensland 4006
Australia

Dr. Chen Bo-Quan
Institute of Virology
Chinese National Center for
Preventive Medicine
100 Yiung Xiug Jie, Xuan Wu Qu
Beijing 100052
Peoples Republic of China

Dr. Stephen Bowen
Associate Professor
Wistar Institute
36th and Spruce Streets
Philadelphia, PA 19104

Dr. Michael W. Brandriss
Professor of Medicine
Infectious Disease Unit
Department of Medicine
University of Rochester
Rochester General Hospital
1425 Portland Avenue
Rochester, NY 14621

Dr. Walter E. Brandt
Assistant Chief
Department of Virus Diseases
Walter Reed Army Institute
of Research
SGRD-UWF-E
Washington, DC 20307-5100

Dr. Carolyn M. Brineman
Research Scientist
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Margo A. Brinton
Associate Professor
Wistar Institute
36th and Spruce Streets
Philadelphia, PA 19104

Dr. Fred Brown
Wellcome Biotechnology Ltd.
Pirbright, Surrey
England
United Kingdom

COL Don Burke
Chief
Department of Virus Diseases
Walter Reed Army Institute
of Research
Washington, DC 20307-5100

M. Jeanne Burrous
Microbiologist
Department of Virus Diseases
Walter Reed Army Institute
of Research
Washington, DC 20307-5100

U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID)

INTERNATIONAL WORKSHOP ON THE MOLECULAR BIOLOGY OF FLAVIVIRUSES
Fort Detrick, Frederick, Maryland
November 29 - December 1, 1984

Participants

Dr. William P. Allen
Virology Program Officer
Microbiology and Infectious Diseases Program
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Westwood Building, Room 736
Bethesda, MD 20205

Janice Amato
Associate Professor
Wistar Institute
36th and Spruce Streets
Philadelphia, PA 19104

LTC Arthur Anderson
Principal Investigator
Airborne Diseases Division
US Army Medical Research Institute of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Fakhry A. Assaad
Director
Division of Communicable Diseases
World Health Organization
1211 Geneva 27
Switzerland

Michael A. Balady
Immunologist
Disease Assessment Division
US Army Medical Research Institute of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. William H. Bancroft
Director
Division of Communicable Disease and Immunology
Walter Reed Army Institute of Research
Washington, DC 20307-5100

Curtis Bartz
Head, Department of Virology
Naval Medical Research Unit 2
Jakarta Detachment, Indonesia
APO San Francisco, CA 96350

Dr. Roy Barzilai
Medical Division
US Army Medical Research Institute of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. William R. Beisel
Special Assistant to the Surgeon General for Biotechnology
US Army Medical Research and Development Command
Fort Detrick
Frederick, MD 21701

Joy Miller Beveridge
Laboratory Technician, Microbiology
US Army Medical Research Institute of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Participants

An expected immediate outcome of the cloning and sequencing of the dengue genome will be the improved characterization of the dengue virus molecular structure. Currently available diagnostic tests have been inadequate to resolve important epidemiological issues related to the pathogenesis of dengue hemorrhagic fever. This precise molecular characterization of dengue viruses is therefore of critical importance for development of a new generation of diagnostic tests, as well as providing a molecular basis for a rational approach to second-generation vaccines.

There was general agreement that lack of understanding of virtually all aspects of dengue virus attenuation prevents development of a rational strategy for selection of attenuated virus. Both the clonal selection and serial passage methods are empirical. Tests in man are virtually the only reliable marker of adequate attenuation (although, to date, small plaque size and temperature sensitivity might be correlated). This being the case, the Committee recommended that efforts be made to characterize successful and unsuccessful vaccines. These include: fingerprinting all vaccine-parent pairs, and studies on permissive host cells for vaccine versus parental virus replication. Greater advantage may be made of *in vitro* infection of human monocytes, and studying differences in replication kinetics. Comparative studies of the S-1 and Mahidol University dengue-2 vaccines in an identical host population may be necessary to determine if there are important differences in reactogenicity and immune responses. Studies on the composition of passage selection vaccines should be done.

RECOMBINANT DNA TECHNOLOGY AS AN APPROACH FOR PRODUCING DENGUE VACCINES:

BACKGROUND:

Cloning and sequencing of a flavivirus genome has been accomplished by Rice et al. using yellow fever virus. Some work has been done with St. Louis encephalitis at Fort Collins, Colorado. The genes coding for the structural polypeptides of flavivirus virions are located at the 5' end of the positive sense RNA genome. Insertion of this region of the yellow fever genome into vaccinia virus has resulted in expression of antigens that have been detected by reference yellow fever antibodies in hyperimmune mouse ascitic fluid. It is not yet known if protective antibodies could be produced *in vivo* by this method.

The Steering Committee recommends that SAGE encourage applications from laboratories having appropriate expertise to assist WHO in applying recombinant DNA technology to dengue fever virus vaccine. Other flaviviruses would be acceptable (e.g., Japanese encephalitis) if they would facilitate development of dengue vaccines.

RECOMMENDATIONS:

1. Cloning and sequencing of the entire genome of each of the four serotypes of dengue virus.
2. Expression of the clones in replicating vectors.
3. Use of monoclonal antibodies to detect the desired epitopes or antigens.
4. Trial infection of animals with vectors to determine if antibodies are produced *in vivo*.
5. Generation of additional monoclonal antibodies to serotypes 1, 3, and 4 that will identify antigens that will induce neutralizing or other antibodies that will be protective.
6. Use of nonstructural antigens as vaccines, since at least one nonstructural antigen (GP 48) appears to induce protective antibodies.

The Thai dengue-1 candidate vaccine virus has been passaged 43 times in PDK cells, is temperature sensitive, and doesn't replicate in monocytes *in vitro*. Because of the satisfactory results with the Thai dengue-2 vaccine prepared in PDK cells, the dengue-1 candidate should be tested in man.

The U.S. dengue-3 live virus vaccine candidate has been passaged and plaque purified in C6/36 *Aedes albopictus* cell culture. Passage in C6/36 cells resulted in small plaque forming, temperature sensitive virus. Final passages were performed in certified fetal rhesus lung cells. The production seed has passed neurovirulence safety testing in monkeys, and the vaccine (produced by additional passage) induced HI and N antibodies in monkeys. The virus was not as stable (in terms of infectivity) as the other serotypes, and additional stabilizers were required to maintain the vaccine strain infectivity.

The Thai dengue-3 candidate vaccine virus was passaged through *T. ambionensis* mosquitoes, and found not to replicate in PDK cells. Since primary green monkey kidney cells (PGMK) supported replication of the virus, they were used to passage the virus 50 times. The vaccine candidate is a small plaque forming virus that induced HI and N antibodies in monkeys, and is avirulent for mice.

The first U.S. dengue-4 vaccine, passaged 35 times in PDK cells and twice in FRhL cells, caused seroconversion in only 2 of 5 volunteers. Virus recovered from the two volunteers who seroconverted exhibited characteristics of the vaccine virus. Because of low immunogenicity, this virus was eliminated from further consideration. Dengue-4 vaccine viruses currently being considered are (1) a 50 PDK passaged virus, and (2) a Caribbean strain of dengue-4 virus passaged in PDK cells. Prototype dengue-4 antibodies do not neutralize the Caribbean strain as well as Caribbean strain antibodies neutralize the prototype virus.

The Thai dengue-4 vaccine candidate has been passaged twice through *Aedes aegypti* mosquitoes and then 43 passages in PDK cells. The virus produced very small plaques, was temperature sensitive, and replicated in monocytes.

DISCUSSION OF LIVE VIRUS VACCINE STRATEGY:

The present strategies of selecting temperature sensitive, small plaque forming viruses as vaccine candidates has produced greatly varying results: seroconversion with some symptoms, low rates of seroconversion, or severe symptoms associated with unmodified dengue fever. Whatever the reasons for this variation, these characteristics have not had predictive value for the success of a vaccine. It was proposed that simple virus passage in PDK cells, as was done with dengue-2 virus, will produce a stable virus that is sufficiently attenuated yet immunogenic in humans. It remains to be determined whether this approach will also be successful with the dengue-1 and dengue-4 candidate viruses that were passaged in PDK cells in Thailand. Dengue-3 virus apparently does not replicate in PDK cells, and the evaluation in humans of dengue-3 passaged in insect cells (U.S.) and PGMK cells (Thailand) has yet to be done.

Three proposals have been approved thus far, and additional ones will be solicited through advertisement to laboratories having capabilities in the areas to deal with the strategies recommended at the end of this report.

REVIEW OF LIVE VIRUS VACCINES AND DISCUSSION OF LIVE VIRUS VACCINE STRATEGY:

The Thai dengue-2 live virus vaccine was prepared by 50 passages in primary dog kidney (PDK) cells. Following safety testing and approval for human trials, the vaccine was inoculated into male volunteers in North Thailand, where very few *Aedes aegypti* and limited circulation of dengue viruses are found. Seroconversion to dengue-2 occurred in all 10 volunteers without significant symptoms (e.g., no fever; some headache [5/10], eye pain [1/10], and mild leukopenia 10 days postinoculation). Neutralization (N) antibody tests with all four dengue virus serotypes on sera obtained at 30 days exhibited titers which were specific for dengue-2 virus. It appeared that 3 of the 10 volunteers may have been flavivirus nonimmunes. It was recommended that more virus isolates from the volunteers be obtained and characterized to fully describe the course of vaccine virus infection in humans, as well as to determine the growth characteristics of the isolated viruses. In addition, it would be important to determine if the vaccine virus could be transmitted by mosquitoes.

A dose-response experiment will be done next to determine the minimum dose of vaccine virus required to produce seroconversion in volunteers. This vaccine appears to be a successful candidate for testing in other countries, especially where infection-enhancing antibodies produced by previous flavivirus infections would not be present to react with the vaccine virus to increase its effectiveness. Will it be effective in flavivirus nonimmunes?

The WRAIR dengue-2 vaccine produced in fetal rhesus lung cells produced mild symptoms in 20% of the recipients, yet viremia occurred at a later time in the recipients than the Thai vaccine. There was 90% seroconversion in flavivirus (yellow fever vaccine) immune individuals and 61% in nonimmunes. The yellow fever immunes maintained their N and hemagglutination-inhibition (HI) antibody titers, whereas the nonimmunes did not even respond by HI, and lost their N antibodies by 6 months. The yellow fever immunes produced IgM followed by IgG, whereas the nonimmunes produced very little, if any, IgG after the IgM was detected. It was suggested that the yellow fever antibodies made the vaccine virus more efficient, possibly by forming infectious immune complexes that entered cells (e.g., monocytes) via their Fc receptors. The virus did not revert in people or upon passage in mosquitoes infected either by inoculation or by feeding on volunteers. Vaccine virus did not infect mosquitoes as efficiently as the wild virus.

The dengue-1 vaccine candidate produced in fetal rhesus lung cells in the United States was also a small plaque temperature sensitive virus, but it produced unmodified dengue fever in the first two volunteers and was eliminated from further testing. For this virus, these laboratory markers served no predictive value in terms of attenuation.

TITLE: Report of Dengue Steering Committee, Scientific Advisory Group (SAGE), World Health Organization, USMARIID, Fort Detrick, Frederick, MD 21701

CHAIRMAN: Natth Bhamarapravati
D.S. Burke, S.B. Halstead, E. Winner, and W.E. Brandt (Rappateur)

REPRESENTATIVES FROM SAGE: F. Assaad and P.K. Russell

REPRESENTATIVE FROM PAN AMERICAN HEALTH ORGANIZATION: F. Pinheiro

DISCUSSANTS: S. Yoksan, W.H. Bancroft, K.H. Eckels, J.B. Moe, and E.G. Westaway

GUESTS: J. Blok and V. Churdboonchart

BACKGROUND:

WHO has agreed to assist in coordinating the efforts of many laboratories working on new methods for vaccine development. Dengue fever is one of five major infectious disease problems of bacterial or viral origin of interest to SAGE. WHO has acquired funds, and is continuously seeking additional funds, to increase the rate of progress and to mobilize the efforts of existing laboratories having ongoing programs contributing to vaccine development. Other areas of interest would be the testing of new vaccines and the transfer of new technology to developing countries.

PROBLEM:

Live virus vaccine development for the four serotypes of dengue fever virus has been very difficult, and experimental vaccines exist only for dengue type 2.

PROGRESS:

The first meeting of the Dengue Steering Committee in February 1984 (Bangkok) considered second-generation dengue vaccines that could be created from recombinant DNA technology. (Live virus vaccine work would continue to be evaluated). The Steering Committee recommended that (1) replicating immunogens will probably be needed for long-lasting protection; (2) a tetravalent vaccine would be desirable because of possible sensitization by one serotype vaccine causing more severe disease with other serotypes of dengue; and (3) infants may not be immunized before 6 months of age because of maternal antibody.

Of the approaches listed in the committee report, SAGE expressed interest in (1) biochemical definition of epitopes, with attention being directed at those involved in neutralization and enhancement; (2) cDNA to obtain genes coding for the envelope glycoprotein and, if possible, specific epitopes; (3) consideration of self-replicating vectors; and (4) clone serologically related 17D vaccine yellow fever virus, since it might be possible to use it as a vector. In addition, comparisons of the genomes of wild and vaccine yellow fever viruses should help reveal the molecular basis for virulence or attenuation.

Report of the Dengue Steering Committee

TITLE: Summary of Round Table Discussion. Session III - Future Vaccine Strategy

AUTHORS: P.K. Russell, F.A. Murphy, and J.M. Dalrymple

ADDRESSES: Fitzsimons Army Medical Center, Aurora, CO 80045; Division of Viral Diseases, Centers for Disease Control, Atlanta, GA 30333; and Department of Viral Biology, Virology Division, USAMRIID, Fort Detrick, MD 21701

TELEPHONE: (301) 663-2665

Reviews of the advantages and disadvantages of existing and experimental vaccines highlighted this session. Antigenic variation necessitating multivalent vaccines can pose a problem, as illustrated by current Japanese encephalitis virus vaccines. Even safe and effective vaccines of long standing, such as the yellow fever 17D vaccines, still have stability and shelf life limitations as well as problems associated with the production of large amounts of vaccine on short notice. Experimental live-attenuated dengue vaccine candidates that were selected on the basis of laboratory and animal markers have now been tested in humans. The human response varied considerably suggesting that presumed markers of attenuation were not absolute and multiple host factors, including pre-existing flavivirus immunity, play a major role in determining infection and immunization with live virus vaccines.

A vigorous discussion of the potential molecular approach to flavivirus vaccines resulted in the opinion that the only valid markers of a live virus vaccine response in humans were obtained by testing in humans. Dr. Scott Halstead "challenged" the molecular virologists to put their technology to work deciphering genetic markers of attenuation, or alternatively, determinants of virus virulence. Such experiments may prove difficult because where virulent-attenuated virus pairs exist (such as with virulent yellow fever and the attenuated 17D vaccine), many genotypic and phenotypic changes unrelated to virulence are expected.

The application of molecular "state-of-the-art" technology to the problems associated with flavivirus vaccine development was expressed as a concluding consensus. A more systematic approach to flavivirus vaccine research combining both molecular and classical methodology predicts success, and much of the collaboration required was initiated at this International Workshop on the Molecular Biology of Flaviviruses. This must be intended for "general" consumption.

PRESENTATION OF THE IMMUNOGEN

In order for a protein molecule to efficiently initiate the immune response, it is essential that it is presented correctly, so that all the components of the immune system are stimulated[6]. Several cells can act as antigen presenting cells, but the most important is the macrophage. Macrophages preferentially take up proteins in a polymeric form and this observation correlates with the known poor immunogenicity of many viral protein monomers, compared to their high immunogenicity as polymers or within virus particles. In order for the macrophage to present the protein antigen, it is first adsorbed, then "processed" and finally oriented on the plasma membrane in association with the correct antigens coded by the MHC complex. Therefore, potential immunogens should be polymeric and have a structure capable of being oriented correctly in the macrophage. In addition, they should contain sequences which allow correct macrophage processing and association with antigens of the MHC complex.

INTERACTIONS WITH HELPER T CELLS

The helper T cells are essential components in the immune system as they not only control the activity of other T cells but also stimulate the antibody-producing B cells. In addition, helper T cells also are involved in determining which antigenic determinants are dominant. As the helper T cell binding region has been shown for some proteins to be different from that of antibody binding, this additional component must also be included in an immunogenic molecule.

UNDESIRABLE CHARACTERISTICS

As suppressor T cells can greatly reduce the immune response to an immunogen, the determinants which recognise them should obviously be removed from the immunogen. Similarly determinants which induce immune enhancing antibodies should also be avoided. This is of particular importance if these determinants are also found on other similar viruses. Finally, regions of the molecule which infer thermal instability or lead to proteolytic degradation could, at least in theory, be removed.

CONCLUSIONS

1) Future vaccines against RNA viruses should be derived from genetic material based on DNA.
2) Good immunogens are complex polymeric molecules with several functions.
3) Recombinent DNA technology should be able to construct such molecules with all the necessary functions while removing those with undesirable properties.

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TITLE: Designing new vaccines against enveloped RNA viruses.

AUTHORS: John R. Stephenson.

ADDRESS: Vaccine Research and Production Laboratory, C.A.M.R., Porton Down, SALISBURY, U.K.

TELEPHONE: 0980 - 610391

INTRODUCTION

During the last few years, major developments in immunology and molecular biology have enabled new methods to be developed for the production of vaccines against viral diseases and have also facilitated an understanding of what should be required of a good immunogenic vaccine preparation. This presentation will concentrate on two aspects of vaccine design. Firstly, the inherent genetic instability of RNA molecules; and secondly, the structural criteria that a potential vaccine should meet in order to accurately and efficiently stimulate the hosts immune response.

GENETIC INSTABILITY IN RNA GENOMES

All RNA viruses (with the exception of those employing a reverse transcriptase) can only replicate by asymmetric transcription from a single strand. They are thus denied most of proof-reading mechanisms available to DNA molecules for correcting errors during polynucleotide chain elongation. The transcriptional error rate for the bacteriophage phage Qß has been calculated at 3×10^{-4} and compared to that of $10^{-9} - 10^{-10}$ obtained from DNA phages[1]. Assuming no corrective features, these figures imply that for a genome of 4.5 kb (e.g. Qß) only 25% of the molecules are error free. When protein mutation rates for RNA viruses have been calculated[2], an overall mutation frequency of $10^{-1.5}$ has been calculated. This means that for the "average" RNA virus with a 10 kb genome, over 10% of all progeny carry a mutation. However, such high mutation rates lead to the absurd conclusion that RNA viruses emerged only after the Second World War! Most probably RNA viruses can change very rapidly but usually oscillate their genetic material around an average RNA sequence.

RNA MUTATION DURING PERSISTENT INFECTIONS

All live attenuated virus vaccines depend on their ability to replicate in the host in order to produce an immune response. Furthermore, the longevity of this response may well depend on the ability of the vaccine strain to induce a persistent infection in the host. However, several studies have demonstrated that mutation rates may be accelerated during persistent viral infections[3].

IMPLICATIONS OF RAPID EVOLUTION FOR VACCINE DESIGN

A genetically unstable genome could have far reaching effects on the efficacy and safety of conventional vaccines against RNA viruses. For example, attenuated strains could revert to neurovirulence as has been demonstrated for poliovirus[4]. Avoidance of the Immune system through antigenic change, induction of suppressor epitopes and Immune enhancement epitopes could also result. Moreover, a change in organ tropism could result, such as observed for measles virus in SSPE.

THE STRUCTURE OF THE ANTIBODY BINDING SITE

One of the decisions in determining the design of a new vaccine is whether to base that design on native protein molecules or synthetic peptides representing antibody sites. A key feature in the decision is whether the topological confirmation of that site is sequentially or spacially determined. The detailed antigenic structure of several proteins has been recently reviewed[5], and the general consensus seems to be that some sites are sequential in nature and others are conformational.

Steve Harrison
Technician
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Shirm Hasty
Technician
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Franz X. Heinz
Institute of Virology
University of Vienna
Kinderspitalgasse 15
A-1095 Vienna
Austria

William E. Houston
Senior Industrial Microbiologist
Engineering and Economics
Research, Inc.
20251 Century Boulevard
Germantown, MD 20767

John W. Huggins
Research Biochemist
Virology Division
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

COL David L. Huxsoll
Commander
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Bruce Innis
Investigator
Department of Virus Diseases
Walter Reed Army Institute
of Research
Washington, DC 20307-5100

Dr. Robert Janssen
Postdoctoral Fellow
Department of Microbiology
University of Pennsylvania
School of Medicine
Philadelphia, PA 19151

Dr. Barbara Johnson
Biochemist
Division of Vector-Borne Viral
Diseases
Centers for Disease Control
P.O. Box 2087
Fort Collins, CO 80522

Dr. Bruce K. Johnson
Deputy Director
Virus Research Centre
Box 20752
Nairobi, Kenya

Dr. Meir Kende
Microbiologist
Virology Division
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

CPT Alan D. King
Aerobiology Division
US Army Medical Research Institute
of Infectious Diseases
Building 1412
Fort Detrick
Frederick, MD 21701

Dr. Srisakul Kliks
National Research Council
Postdoctoral Fellow
Department of Virus Diseases
Walter Reed Army Institute
of Research
SGRD-UWF-E
Washington, DC 20307

Dr. Fred K. Knauert
Microbiologist
Medical Division
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Ana Ines Kuehne
Microbiologist
Virology Division
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Ching-Juh Lai
Head, Molecular Viral Biology
Section
Laboratory of Infectious Diseases
National Institute of Allergy and
Infectious Diseases
Building 7, Room 26
National Institutes of Health
9000 Rockville Pike
Bethesda, MD 20205

Rob Lanciotti
Research Chemist
Virology Division
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Kingkarn Laohathai
Mahidol University
Ramathibodi Hospital
Rama IV Road
Bangkok 10400
Thailand

CMDR Larry Laughlin
Naval Medical Research Unit 2
Detachment Jakarta, Indonesia
APO San Francisco, CA 96356

James LeDuc
Chief, Department of Epidemiology
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Richard M. Lewis
Research Chemist
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Carol Linden
Research Biologist
Virology Division
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Bruno J. Luscri
Microbiologist
Virology Division
Department of Antiviral Studies
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

CPT William H. Lyerly, Jr.
Immunologist
Armed Forces Medical Intelligence Center
Building 362
Fort Detrick
Frederick, MD 21701

Dr. Erich R. Mackow
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, MD 20205

Yoshihiro Makino
Visiting Associate
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, MD 20205

Peter Markiewicz
Research Associate
US Army Medical Research Institute of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Louis Markoff
Senior Investigator
Laboratory of Infectious Diseases
Building 7, Room 26
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, MD 20205

Dr. Peter Mason
Postdoctoral Research Associate
Department of Biochemistry
University of Massachusetts
Amherst, MA 01003

Dr. Thomas L. Mason
Associate Professor
Department of Biochemistry
University of Massachusetts
Amherst, MA 01003

Dr. James H. Mathews
Biologist
Immunochemistry Branch
Division of Vector-Borne Viral Diseases
Centers for Disease Control
P.O. Box 2087
Fort Collins, CO 80522-2087

Dr. Phyllis McAda
Postdoctoral Research Associate
Department of Biochemistry
University of Massachusetts
Amherst, MA 01003

Jack McCown
Laboratory Technician
Department of Virus Diseases
Walter Reed Army Institute of Research
Washington, DC 20307-5100

MAJ Kelly T. McKee, Jr.
Research Scientist
US Army Medical Research Institute of Infectious Diseases
SGRD-UIMI
Fort Detrick
Frederick, MD 21701

Dr. James M. Meegan
Medical Division
US Army Medical Research Institute of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Barry R. Miller
Research Entomologist
Division of Vector-Borne Viral Diseases
Centers for Disease Control
P.O. Box 2087
Fort Collins, CO 80522-2087

LTC(P) James B. Moe
Director, Division of Pathology
Walter Reed Army Institute of Research
SGRD-UWP
Washington, DC 20307-5100

Dr. Thomas P. Monath
Director
Division of Vector-Borne Viral Diseases, CDC
c/o Gastroentrology Unit
Massachusetts General Hospital
Boston, MA 02114

Dr. David M. Morens
Department of Tropical Medicine and Medical Microbiology
University of Hawaii School of Medicine
Leahi Hospital
3675 Kilauea Avenue
Honolulu, HI 96816

John C. Morrill
Veterinary Microbiologist
Disease Assessment Division
US Army Medical Research Institute of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Frederick A. Murphy
Director
Division of Viral Diseases
Centers for Disease Control
Atlanta, GA 30333

CPT Ed Nuzum
Veterinarian
Disease Assessment Division
US Army Medical Research Institute of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Julio Barrera Oro
The Salk Institute at the
US Army Medical Research Institute of Infectious Diseases
5596 Parkview Court
Frederick, MD 21701

Edward O'Rourke
Instructor, Division of Infectious Disease
Department of Medicine
Children's Hospital Medical Center
Enders 622
300 Longwood Avenue
Boston, MA 02115

Dr. Radha Padmanabhan
Associate Professor
Department of Biochemistry
University of Kansas Medical Center
39th and Rainbow Boulevard
Kansas City, KS 66103

Dr. Tikki Pang
Associate Professor
Department of Medical Microbiology
Faculty of Medicine
University of Malaya
Kuala Lumpur
Malaysia

William C. Patrick
Program Analysis Officer
US Army Medical Research Institute of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. C.J. Peters
Chief, Disease Assessment Division
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Francisco P. Pinheiro
Regional Advisor on Viral Diseases
Epidemiology Unit
Pan American Health Organization
World Health Organization
525 23rd Street, N.W.
Washington, DC 20037

Dr. Peter G.W. Plagemann
Professor
Department of Microbiology
University of Minnesota Medical
School
Minneapolis, MN 55455

Dr. James S. Porterfield
Sir William Dunn School of
Pathology
University of Oxford
South Parks Road
Oxford OX1 3RE, England
United Kingdom

MG Garrison Rapmund
Commander
US Army Medical Research and
Development Command
SGRD-ZA
Fort Detrick
Frederick, MD 21701

Patricia M. Repik
Microbiologist
Department of Viral Biology
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Charles M. Rice, III
Division of Biology 156-29
California Institute of Technology
Pasadena, CA 91125

COL David Robinson
Project Manager for Biologics
Systems
US Army Medical Materiel
Development Agency
Fort Detrick
Frederick, MD 21701

Dr. John T. Roehrig
Research Microbiologist
Immunochemistry Branch
Division of Vector-Borne Viral
Diseases
Centers for Disease Control
P.O. Box 2087
Fort Collins, CO 80522-2087

BG Philip K. Russell
Commanding General
Fitzsimmons Army Medical Center
Aurora, CO 80045

Dr. Kevin Ryan
Staff Fellow
National Institute of Allergy
and Infectious Diseases
Building 7, Room 22
National Institutes of Health
Bethesda, MD 20205

Dr. Jacob J. Schlesinger
Associate Professor of Medicine
Infectious Disease Unit
Department of Medicine
University of Rochester
Rochester General Hospital
1425 Portland Avenue
Rochester, NY 14621

Dr. R. Walter Schlesinger
Department of Microbiology
University of Medicine and
Dentistry of New Jersey
Rutgers Medical School
P.O. Box 101
Piscataway, NJ 08854

Dr. Alan Schmaljohn
Assistant Professor
Department of Microbiology
University of Maryland
School of Medicine
660 West Redwood Street
Baltimore, MD 21201

Connie Schmaljohn
Microbiologist
Virology Division
Department of Viral Biology
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Randal J. Schoepp
Research Associate
Department of Microbiology
Colorado State University
Fort Collins, CO 80523

Robert McNair Scott
Head, Virology
Naval Medical Research Unit 3
FPO NY 09527
Cairo
Egypt

Thomas W. Scott
Assistant Professor
Department of Entomology
University of Maryland
College Park, MD 20742

Dr. Alexis Shelokov
Director of Vaccine Research
Government Services Division
The Salk Institute
The Johns Hopkins University
School of Hygiene and Public Health
615 North Wolfe Street
Baltimore, MD 21205

Dr. Robert E. Shope
Professor of Epidemiology
Yale Arbovirus Research Unit
Department of Epidemiology and
Public Health
Yale University School of
Medicine
60 College Street
P.O. Box 3333
New Haven, CT 06510-8034

Greg Smith
Laboratory Technician
Medical Division
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Jonathan Smith
Microbiologist
Virology Division
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

COL Richard O. Spertzel
Deputy for Research Management
US Army Medical Research Institute
of Infectious Diseases
SGRD-UIZ-E
Fort Detrick
Frederick, MD 21701-5011

E.H. Stephenson
Chief, Aerobiology Division
US Army Medical Research Institute
of Infectious Diseases
Building 1412
Fort Detrick
Frederick, MD 21701

Dr. John R. Stephenson
Leader, Molecular Biology Group
Vaccine Research and Production
Laboratory
Center for Applied Microbiology
and Research
Public Health Laboratory Service
Porton Down, Salisbury
Wiltshire SP4 OJG, England
United Kingdom

Dr. Victor Stollar
Professor of Microbiology
Department of Microbiology
University of Medicine and
Dentistry of New Jersey
Rutgers Medical School
P.O. Box 101
Piscataway, NJ 08854

Dr. Ellen G. Strauss
Senior Research Associate
Division of Biology 156-29
California Institute of Technology
Pasadena, CA 91125

Dr. James H. Strauss
Professor of Biology
Division of Biology 156-29
California Institute of Technology
Pasadena, CA 91125

Peter L. Summers
Walter Reed Army Institute
of Research
Washington, DC 20307

Ralph F. Tammariello, Jr.
Research Assistant
Arboviral Entomology
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Irmgard Tappert
Research Assistant
Disease Assessment Division
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

May C. Tom
Research Associate
Department of Tropical Medicine
University of Hawaii
3675 Kilauea Avenue
Honolulu, HI 96816

COL Franklin H. Top, Jr.
Director
Walter Reed Army Institute
of Research
Washington, DC 20307

Dr. Dennis W. Trent
Chief, Immunology Branch
Division of Vector-Borne Viral
Diseases
Centers for Disease Control
P.O. Box 2087
Fort Collins, CO 80522

CPT Michael A. Ussery
Microbiologist
Virology Division
Department of Antiviral Studies
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Vik Vakharia
Research Associate
Department of Biochemistry
University of Kansas Medical Center
39th and Rainbow Boulevard
Kansas City, KS 66103

Dr. Sundararajan Venkatesan
Microbiologist
Office of the Scientific Director
National Institute of Allergy and
Infectious Diseases
Frederick Cancer Research Facility
Frederick, MD 21701

Dr. Vance Vorndam
Virologist
Immunochemistry Branch
Division of Vector-Borne Viral
Diseases
Centers for Disease Control
P.O. Box 2087
Fort Collins, CO 80522-2087

Dr. Douglas M. Watts
Microbiologist
Division of Virology
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick
Frederick, MD 21701

Dr. Edwin G. Westaway
Department of Microbiology
Monash University
Clayton, Melbourne
Victoria 3168
Australia

Dr. Eckard Wimmer
Department of Microbiology
State University of New York
Stony Brook, NY 11790

Dr. Charles Wisseman
Professor and Chairman
Department of Microbiology
University of Maryland School
of Medicine
660 West Redwood Street
Baltimore, MD 21201

CDR Owen L. Wood
Virologist
Aerobiology Division
US Army Medical Research Institute
of Infectious Diseases
Building 1412
Fort Detrick
Frederick, MD 21701

Dr. Kotaro Yasui
Senior Researcher
Department of Microbiology
Tokyo Metropolitan Institute
for Neurosciences
2-6 Musashidai
Fuchu-City, Tokyo 183
Japan

Dr. Sutee Yoksan
Instructor in Pathology
Faculty of Medicine
Department of Pathology
Ramathibodi Hospital
Bangkok 10400
Thailand

Dr. Bangti Zhao
Visiting Associate
National Institute of Allergy
and Infectious Diseases
National Institutes of Health
Building 7, Room 22
9000 Rockville Pike
Bethesda, MD 20205

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